Leaching of pathogens from manure to drainage water - assayed using classic and DNA/mRNA based methods

Publication date:
2009

Document version
Publisher's PDF, also known as Version of record

Citation for published version (APA):
III International Conference on Environmental, Industrial and Applied Microbiology

Fostering Cross-disciplinary Applied Research in Microbiology and Microbial Biotechnology

BioMicroWorld 2009

2 - 4 December 2009
Lisbon, Portugal

http://www.formatex.org/biomicroworld2009
BioMicroWorld-2009
III International Conference on Environmental, Industrial and Applied Microbiology
Lisbon, Portugal, 2-4 December 2009
http://www.formatex.org/biomicroworld2009

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Activated carbon production from brewer’s spent grain lignin

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Alternative method for biological airborne agents detection in only few hours / Innovative microbial air sampler

Amino acid uptake profiling of *Streptomyces lividans* batch fermentations

ANN-based Software Sensor for Emulsification Activity Estimation in Biosurfactant Production Process by *Candida lipolytica* UCP 0998

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Antifungal potential of *Cladosporium cladosporioides* (Fres) De Vries metabolites in reduction of coffee rust (*Hemileia vastatrix* Berk & Br.)

Assessment of the use of biological material on technological development – a patent approach

Bacteria exhibiting antimicrobial activities; screening for antibiotics and the associated genetic studies

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Biological detoxification of different hemicellulosic hydrolyzates using *Isatchenka orientalis* CCTCC M 206097 yeast

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A new Bacteroides host strain for the detection of bacteriophages indicating human faecal contamination in water

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Faecal contamination of water used as drinking water, for recreation or for food production may lead to waterborne diseases and economic losses. It is therefore important to protect water resources. In many countries, the faecal indicator organisms, E. coli and enterococci, are used for the description of the microbiological quality of water. Based on these organisms it is not possible to distinguish between human and animal faecal pollution. Such discrimination, however, is crucial for water management because it is generally assumed that pathogens with impact on human health are more frequently transmitted through human faecal pollution. To differentiate between various types of contamination and to trace their origin, effective and easy to handle methods should therefore be established to determine whether the water is contaminated by human or animal faeces. In this study a new Bacteroides host strain termed Bacteroides thetaiotaomicron ARABA 84 was isolated which is specific for bacteriophages of human origin. Moreover, the strain is applicable for the detection of human faecal contaminations in water.

The specificity of the strain was determined by examination of human and animal wastewater. Bacteriophages were exclusively found in human wastewater and never detected in animal waste. Analysis of surface water samples showed that bacteriophages infecting the novel host strain are present in the environment. In river water samples taken after a wastewater treatment plant, bacteriophages of human origin were present in all samples analysed.

In addition, the new host strain was used for investigation of a spring located in the north western part of Switzerland. The strain, which is part of a karst aquifer, is vulnerable for faecal contamination because of hydrogeologic characteristics. Previous studies showed that after heavy rainfall the spring was contaminated with E. coli, enterococci and even pathogens. Bacteriophages of the newly isolated Bacteroides host strain were present after three rain events thus indicating the presence of human faecal contaminations. Based on these results, it can be concluded that the new host strain, Bacteroides thetaiotaomicron ARABA 84, is a promising tool for the use in microbial source tracking.

Keywords: bacteria; contamination; indicator; pollution; source tracking

Agrobacterium radiobacter / Agrobacterium tumefaciens human isolates form a sub-population distinct from the environmental strains

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The genus Agrobacterium groups environmental bacteria, some of them are phytopathogenic causing crown gall. Agrobacterium is also recognized as an agent of human opportunistic infections even if its pathogenicity remains to be investigated. Taxonomy and nomenclature is controversial in the genus Agrobacterium. As a rule, clinical strains are named Agrobacterium radiobacter whereas Agrobacterium tumefaciens is preferred for the phytopathogenic strains. The complete genomic sequence of A. tumefaciens and A. radiobacter is available. Their genomes are composed of a large circular chromosome associated with a linear one.

We proposed phylogenetic and Multi Locus Sequence Analyses (MLSA) in order to study the population structure of clinical and environmental isolates of A. tumefaciens / A. radiobacter. Forty-two phytopathogenic and non phytopathogenic plant isolates, 45 clinical isolates and reference strains of the species A. radiobacter and A. tumefaciens are investigated. A MLSA scheme is proposed for the first time for this bacterial species. The scheme was based on the partial sequences of 6 housekeeping genes (atpD, zwf, rpoD, groEL, shuK and rpoD) distributed around the large circular chromosome. Clonal complexes were defined using e-burst program and minimum spanning treeing. Each major clonal complexes contained either plant or human isolates. The phylogenetic analyses were performed by maximum likelihood method implemented with the General Time Reverse model. Tree reconstruction based on the concatenated sequences showed the existence of two major robust clades corresponding to the environmental and clinical strains, respectively. Phylogeny and population structure segregated the clinical and environmental sub-populations. This organization suggested the adaptation of clades and/or clonal complexes to man and to the opportunistic pathogen behaviour.

Keywords: bacteria; contamination; indicator; pollution; source tracking
Antimicrobial activity of soft coral *Sinularia compressa* from Hengam Island, the Persian Gulf

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Antimicrobial activity was examined in the soft coral, *Sinularia compressa*, from Hengam Island, the Persian Gulf. Extraction and assay protocols were developed to identify antimicrobial activity in some fractions of extract. Detection was determined by disc assay method with antibiotic as control, using two gram positive bacteria, *(Staphylococcus aureus* and *Bacillus* sp) and four gram negative bacteria, *(Pseudomonas* sp, *Klebsiella* sp, *Salmonella* sp and *Shigella* sp). TLC, ninhydrin and vanillin/sulfuric acid reagents and GC-MS were used to isolate, identify and characterize metabolites in fractions. The results demonstrate that some fractions of *S. compressa* extract such as butanol and ethyl acetate fractions exhibited appreciable antimicrobial activity on some bacteria, while some fractions had little or no antimicrobial activity. Acetone and hexane fractions had no antimicrobial activity on *Pseudomonas* sp, *Klebsiella* sp, *Salmonella* sp and *Shigella* sp, while antimicrobial activity of ethyl acetate fraction on *Staphylococcus aureus* and butanol fraction on *Pseudomonas* sp was considerable.

Keywords antimicrobial activity; *Sinularia compressa*; Hengam Island; Persian Gulf

Aquatic hyphomycetes: what can they tell us about stream ecological integrity?

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Aquatic hyphomycetes are a group of freshwater fungi, composed by the anamorphs (asexual stages) of ascomycetes and basidiomycetes. Even though this is a phyllogenetically heterogeneous group, there are common features to most species: (a) they are saprophytic, using submerged leaves and wood as substrates and sources of carbon, (b) they can retrieve nutrients (e.g. N and P) from both the water column and the organic substrates, (c) they inhabit preferentially well aerated and turbulent freshwater systems, and (d) they produce numerous conidia (asexual spores) with distinctive shapes and sizes (Fig. 1). This ecologically homogeneous group of microorganisms (decomposers) constitutes a key component of small woodland streams, where the basis of aquatic food webs is litter decomposition. However, human induced changes on water quality or on riparian vegetation can affect the activity and community structure of these organisms, with consequences on litter decomposition and aquatic food webs. It is therefore urgent to deep our understanding on how anthropogenic activities affect these microorganisms; if their responses to a given stress factor are predictable this might even help us to detect early impairments to freshwaters using fungal activity or structural variables as bioindicators.

Here we assessed the effect of stream water nutrient enrichment (cultural and experimental) and substitution of native forests by eucalyptus plantations on (a) fungal biomass built up (determined after extraction of the membrane lipid ergosterol from a given amount of colonized litter), (b) conidial production rate (determined after counting the number of conidia produced after incubating a given amount of colonized litter under laboratory conditions) and (c) community structure (determined after identification and counting of conidia) of aquatic hyphomycetes associated with submerged litter.

Increases in dissolved nutrients (100–1100 μg/L) in stream water generally increased (15–100%) fungal biomass built up, although significant difference between reference and impacted sites depended on litter identity. Conidial production rate was stimulated by nutrient enrichment, but more for nutrient poor substrates (e.g. balsa veneers, 7–25 times the peak value at the reference site) than for nutrient rich substrates (e.g. alder litter, 2–4–times). Even though there was a tendency for nutrient enrichment to stimulate fungal production, the extent of this stimulation was species specific, which resulted in changes in community structure. However, these changes were more marked for communities associated with fast decomposing litter (e.g. alder litter) since here time was not an important factor, than for those on slow decomposing litter (e.g. oak litter) where time, and ecological succession, has a strong effect on structuring fungal communities which overrides the effect of nutrient enrichment.

Substitution of native forests by eucalyptus plantations in Central Portugal led to increased instream organic matter storage (due to slower decomposition rates of eucalyptus leaves, higher amount of bark, and higher litter fall during summer when flow is low) and increased water temperature (due to open canopies), which resulted in higher fungal biomass built up and higher conidial production in eucalyptus streams than in reference streams. However, the fungal community structure was most affected by forest change with a reduction of 20–25% in the total number of species.

The activity and community structure of aquatic hyphomycetes were sensitive to both anthropogenic factors studied here, suggesting that they can give important information on both the functional and structural components of ecological integrity of freshwaters.

Keywords aquatic hyphomycetes; biomass; community structure; eucalyptus; eutrophication; sporulation

![Fig. 1 Conidia of aquatic hyphomycetes. From leaf to right: Casariella splagonum, Lemoniera terrestris, Tetrachaetum elegans (2), Tricladium chaetocladium, Tomularia aquatica (4), Clavariopsis aquatica, Helicomyces lagunensis (3), and Tetrachaetum marshallianum. Conidia are not to scale.](image-url)
Archaea from Algerian Hypersaline Environments producing Archaeocins

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This study aimed to characterize and identify archaeal strains producing bacteriocin-like substances, with a focus on halophilic species.

Over 150 microbial strains have been isolated from different hypersaline environments of Algeria: salt lakes, seawater, seaweed, and salted foods. Fifty per cent presented an antagonistic activity towards at least one of the other strains isolated along this study, while most of these were also capable of inhibiting the growth of eubacterial reference strains. Complete inactivation or significant reduction in activity was observed after treatment of active cell-free supernatants with trypsin, pepsin or papain, thus suggesting production of bacteriocin-like substances.

Eight archaeal isolates were identified by PCR amplification of 16S ribosomal DNA. Purification of the active substances from four of these strains revealed that they are short hydrophobic peptides, presumably microhalocins. Growth and production of potent microhalocin was optimized for one of the strains, SM5. Different conditions of temperature, pH, salt concentration and a variety of carbon sources were tested. An optimal artificial medium was defined. Culture and production of active compounds was shown to be possible in bioreactors, using food industry by-products (molasses and lactoserum) as growth media. Growth was maximal in molasses supplemented with yeast extracts, thus suggesting possible waste recycling for future production of the microhalocin.

The microhalocins exhibited original features, such as a capacity to adsorb onto the surface of producer cells, thus suggesting a cationic nature. This particularity might be correlated with their unprecedented inhibitory activity on several eubacterial strains, with different degrees of specificity. This is the first report that archaeocins, the archaeal pendant of bacteriocins, can inhibit the growth of members of another domain of life, the Bacteria.

Keywords: Saltens, Algeria; halophilic archaea; microhalocins; bacteriocin; antibacterial activity; production; agricultural by-products.

Assessing the viral pollution in Korean water environments by using Integrated cell culture-PCR and real-time PCR

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Enteric viral pollution was studied in Korean water environments (river water, wastewater treatment plant effluent, beach water, sea water) by using Integrated cell culture-PCR (ICC-PCR) and real-time PCR. Water samples were collected five times at water environments near Gangneung City in 2008. The enteric viruses (adenovirus and enterovirus) were detected in all sites. The main location of the viral pollution source was different between ICC-PCR and real-time PCR. ICC-PCR results showed that source of the infectious enteric viruses was located on the upstream of the river whereas the main source of viral pollution seemed to be wastewater treatment plant effluent by using real-time PCR. These results indicate that real-time-PCR may be rapid and sensitive method for detecting viruses, but there is a limit that it can’t assure where the main location of the infectious virus source is, so the methods based on cell culture assay are necessary to make up the deficits of the real-time PCR for assessing the viral pollution.

Supported by granted from BK21 and National Fisheries Research & Development Institute
Automated detection and quantification of total bacteria in liquid samples based on the MPN method

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Quantification of culturable bacteria is a widely used measure not only for water analysis but also for food. Especially in terms of drinking water analysis, testing for microorganisms is strictly regulated by the European Drinking Water Directive including quality criteria for the methods to be used and detection limits (Council Directive 98/83/EC, EN ISO 8199).

The most common method for a total bacteria count is the pour plate method (EN ISO 6222), which can be labour intensive as well as user-dependent in terms of enumeration of visible colonies. Additionally, the fact that hot agar (45 ± 1 °C) is poured on the sample containing microorganisms adapted to psychrophilic environments, may lead to heat shock (Sartory et al. 2008) or simply bad distribution of the sample, if the agar is cooling down too quickly during the mixing process.

The method presented in this study is based on the Most Probable Number (MPN) method which was adapted to comply with the need for a quick and easy screening tool for different kinds of liquid samples as well as varying microbial load. The use of 24 well titer plates instead of tubes for cultivation of bacteria drastically reduces the amount of culture media and also simplifies incubation. Aliquots of 1 ml sample mixed with 1 ml double concentrated media showed to be a good compromise between precision and handling, which can be further simplified by use of dispensers.

Photometric detection of turbidity instead of visual evaluation of bacterial growth avoids misinterpretation of users and provides additional information through absorbance values. Definition of a threshold ensures user-independent determination of microbial growth. Calculation of the MPN itself is done using a program provided by the FDA (U.S. Food and Drug Administration, Blodgett 2003) that also provides basic statistics and allows great flexibility concerning sample volume, number of dilutions and replicates within certain dilutions (Garthright & Blodgett 2003).

Validation criteria for this method were carefully established and evaluated. This includes assessment of applicability for various sample materials, utilisation of different dilution proportions and implementation of varying numbers of replicates per dilution as well as tests for accuracy, repeatability, sensitivity and robustness. Thus, an SOP (Standard Operating Procedure) for the quantification of microbial contamination of water, coloured and turbid aqueous samples was established and can be used as an alternative screening technique to the pour plate method.

Keywords: total bacteria count, MPN, automated detection, quantification

Bacterial sulfate reduction in the oxic zone of acidic gold mine tailings contaminated with arsenic and other metals

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Microbial sulfate reduction (MRS) is instrumental in precipitating metal sulfides and generating alkalinity in mining sites impacted by acid drainage waters. MRS has traditionally been regarded as an anaerobic process and most earlier studies of sulfate-reducing bacteria activity and diversity focused on the reduced subsurface zone of mine tailings. The aim of this study was to assess the activity and diversity of sulfate-reducing microorganisms in the oxidized surface layer of a gold mine tailings deposit heavily polluted with metals.

A multiphasic approach, encompassing molecular, microbiological and geochemical analysis, was used to study bacterial sulfate reduction in the oxic zone of an abandoned gold mine tailings deposit in the Kuznetsk Basin (southwestern Siberia). The leachate produced in the tailings was highly acidic (pH 2) and contained elevated metal concentrations: Fe up to 9,100 mg/l, As up to 1,900 mg/l, Zn up to 351 mg/l, and Cu up to 35 mg/l. The sulfate-reduction rate measured with 35SO4-tracer reached up to 60 nm/cm3/day. Molecular cloning only revealed spore-forming Firmicutes, capable of dissimilatory sulfate reduction, to be present in the tailings. Na Desulfovibrio bacteri a were found by cloning, denaturation gradient gel electrophoresis of 16S rRNA genes (PCR-DGGE), or cultivation. Molecular cloning of the dsrAB gene, a functional gene marker of sulfate reduction, and subsequent phylogenetic analysis, revealed three distinct groups of clones. Two of them have no close cultivated relatives and cannot be assigned to any known taxa containing microorganisms capable of dissimilatory sulfate reduction. The third group branches among Gram-positive Desulfosporosinus. Cultivable Desulfosporosinus-like organisms have been retrieved from the tailings. The 16S rRNA gene sequence homology of isolated phylotypes with known Desulfosporosinus was, however, 90% or lower.

Our study shows that a microbial consortium of active sulfate-reducing bacteria, tolerating extremely high metal and proton concentrations, occurs in the oxic zone of mine tailings. The consortium includes spore-forming sulfate-reducers and most likely new undescribed organisms whose presence can be tracked by dsrAB genes.

We acknowledge support from the Russian Fund for Fundamental Research (Grants 09-04-01256-a and 07-04-90833-mob_st). We are indebted to Alexander Loy for his help with phylogenetic analysis of dsrAB genes.

Keywords: 16S rRNA; bacterial sulfate reduction; Desulfosporosinus; DGGE; dsrAB; mine tailings; molecular cloning; sulfate-reducing bacteria;
Bacterial Trade-off between Antibiotic Resistance and Biological Fitness

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Antibiotics have been widely used in the many fields of human health and agriculture. However, an indiscreet use of antibiotics brings about the increased frequencies of antibiotics resistant bacteria in the environment. Antibiotic resistance in bacterial cells may cause some unexpected results, such as the alterations of phenotypic and physiological characteristics. These bacterial adaptations on antibiotics challenges are called the “biological fitness cost” However, phenotypic and genetic changes associated with antibiotic resistance other than growth-deficit have been poorly characterized. To address in more detail the effect of antibiotics on biological fitness cost, we studied a recent environmental isolate, *Acinetobacter* sp. strain DR1 which was proven to be a diesel oil degrading bacterium. Genome sequencing of strain DR1 is in progress and will be discussed. Rifampicin, a bactericidal antibiotic drug, is routinely used to make an environmental recipient selective on laboratory-conjugation experiment. Accidentally, we noticed that rifampicin-resistant strain DR1 showed substantial loss of quorum sensing signal. Domesticated ampicillin-resistant strain DR1 displayed more dramatic phenotypic changes than rifampicin-resistant cells: complete loss of quorum sensing, loss of swimming and swimming motilities, the poor expression of fimbric, change of membrane fatty acid composition to be more rigid, and decreased capability of hec adenace degradation. Interestingly, the motility of strain DR1 grown next to a streptomycin-producing *Streptomyces griseus* permanently disappeared, where this change was heritable and other phenotypic changes could not be noticeable. Our data demonstrated that each antibiotic has different degree of phenotypic and genetic alteration. Systematic analysis of these genetic alterations in antibiotic-resistant bacteria using proteomics and microarray techniques will be discussed. Our report is significant because we provide important evidence of phenotypic and genetic changes in antibiotic resistant bacteria no matter where this acquisition of antibiotic resistance happens: laboratory-acquired or environment-acquired. Our data also suggested that domesticating environmental isolates should be caution because there are phenotypic variations of antibiotic resistant cells, which could not be noticeable all phenotypic assays are tested.

Bactericidal and amoebicidal activities of the free living amoeba *Willaertia magna*

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*Legionella pneumophila*, the causative agent of Legionnaire’s disease, is a facultative intracellular parasite of some mammalian cells such as macrophages and monocytes. In freshwater environment, the bacterium is known to replicate within protozoan such as ciliates and free-living amoebas. More and more evidences show that free living amoebas are playing a crucial role in the ecology of *Legionella pneumophila*. Hence, in freshwater, high bacterial concentrations are often associated to elevated levels of free-living amoebas and the presence of amoebas is able to trigger the revival of *Legionella* strains that were noncultivable. Although, Fields reported that 13 amoebic species are able to support *Legionella pneumophila*, most of in vitro experiments addressing the bacterium/amoebic interactions have been performed using two genera of amoebae, namely *Acanthamoeba* and *Hartmannella*. We isolated from freshwater two strains of the free-living amoeba *Willaertia magna* (strain c2c Maky and strain d2 Nata) that display a particular resistance toward the bacterium *Legionella pneumophila*. The growth of these strains was not affected when cultured in presence of different strains of *Legionella pneumophila* serogroup 1 (strains Lp Paris, lens and Philadelphia) and no obvious bacterial cytotoxic effects could be observed. At the opposite, when *Legionella pneumophila* strains were co-cultured with the amoeba *Acanthamoeba castellanii* and *Hartmannella vermiformis* (two known vectors of the bacterium) a strong cytotoxic effect towards the protozoan hosts was observed. Moreover, *Willaertia magna* strains were able to inhibit the growth of *Legionella pneumophila* at the opposite to *Acanthamoeba castellanii* and *Hartmannella* vermiformis. These observations highlight the two strains of *Willaertia magna* as particularly resistant to the bacterium *Legionella pneumophila*. Electron microscopy observations show that the occurrence of bacterial replicative phagosomes within *Willaertia magna* c2c Maky is much lower than in other amoebas, i.e., *Acanthamoeba castellanii* and *Hartmannella*. These observations may have important implications for understanding the ecology of *Legionella pneumophila* and highlight the *Willaertia magna* isolated strains as a putative tool in a strategy of biological struggle against the bacterium.

In addition to the bactericidal effect of *Willaertia magna* towards *Legionella pneumophila* we also demonstrate the occurrence of interamoebic phagocytosis inasmuch the free living amoebas *Acanthamoeba castellanii* and *Willaertia magna* were able to phagocyte and digest other amoebic specie such as *Hartmannella vermiformis*. We investigated the putative consequences of this interamoebic phagocytosis on the outcome of *Legionella pneumophila* proliferation. Our results show that *Legionella pneumophila* proliferation is highly dependant on the ability of the predator amoeba to sustain it. To our knowledge it is the first time that the phagocytosis of an amoebic specie by another one is reported. These observations may have important implications for understanding the ecology of *Legionella pneumophila* and highlight the *Willaertia magna* isolated strains as a putative tool in a strategy of biological struggle against the bacterium.


Keywords: Free-living amoebas, *Legionella pneumophila*
Bioconversion of the residue from cachaça production (vinasse) into Saccharomyces and Candida biomass

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The residue (vinasse) formed during the distillation of cachaça, a traditional rum-type spirit produced from sugar-cane in Brazil, is highly harmful if discharged into the environment. One possibility for minimizing the impact of vinasse in soils and waters is to use the residue in the production of microbial biomass for use as an animal feed supplement. This paper reports the results obtained following the fermentation of isolates of Saccharomyces cerevisiae and Candida parapsilosis, originating either from cachaça-distillation units or from fuel alcohol-producing industries, in culture medium containing up to 50% (v/v) of vinasse. Assays were conducted under twelve different culture conditions involving variations in the concentrations of glucose, yeast extract and potassium sulphate, and at different temperatures and pH values. In each case, the microbial biomasses obtained following 168 h of incubation were evaluated with respect to production, productivity, nitrogen content and acid nucleic contents. None of the conditions tested influenced significantly the characteristics of the microbial biomass produced. Of the S. cerevisiae isolates tested, two (VR1 and PE2) originating from fuel alcohol-producing plants were identified as offering the best potential for the industrial production of single cell protein from vinasse.

Keywords: vinasse, microbial protein, bioconversion, Plackett-Burman.

Biodegradation of aromatic amines in a packed bed biofilm reactor

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Sulfonated aromatic amines (SAAs), widely used for chemical synthesis, can be released to the environment by the Chemical Industry. However, the main SAAs pollution sources derive from the synthesis and biodegradation of textile azo dyes. It is well known that microbial azo dye reduction usually generates recalcitrant byproducts such as 4-amino benzenesulfonic acid (4-ABS) or sulfamic acid) or 4-amino-naphthalene sulfonic acid (4-ANS), after the azo-linkage cleavage of Acid Orange 7, or Acid Red 88, respectively. Both SAAs are frequently used as constituents of many azo dyes; thus, in azo dyes degradation processes, 4-ABS and 4-ANS are frequently found. By these reason, a mixture of these aromatic amines was used to evaluate their biodegradation by a bacterial association attached to porous volcanic stone.

The biodegradation process was carried out in an aerobic-packed-bed column continuously fed with a mineral salts medium plus SAAs as the sole nitrogen, sulphur and carbon source. Volumetric loading rates were varied from 5.5 to 50 mg L⁻¹ h⁻¹. SAAs removal efficiencies were calculated after residual amines were measured by liquid chromatography (HPLC) and by Chemical Oxygen Demand (COD).

When the packed bed reactor was operated at values lower than 27 mg L⁻¹ h⁻¹, the bacterial association, composed by five morphological distinct bacterial strains, was able to remove 100% of 4-ABS and 98% of 4-ANS, with a COD removal efficiency of 96%. At higher loading rates, the removal efficiencies decay.

Keywords: 4-amino benzene sulfonate, 4-amino naphthalene sulfonic acid, biodegradation, biofilm.
Biodegradation of organic matter in lake water in different temperature condition (mesocosms experiment)

D. A. Górniaks, J. A. Dunalska, and B. Jaworska

The main subject of the study was to determine the degree to which elevated water temperature in nutrient-poor and nutrient-rich aquatic environments has effect on the intensification primary production and on the organic matter transformations by bacteria. The studies were conducted in experimental conditions. The experiment system consisted of six mesocosms, and a heating and cooling system. In three mesocosms there was water from a eutrophic lake, in another three mesocosms from a mesotrophic lake. The system kept three different temperatures: one was the same as in the lake (20 °C - the control temperature), in two other mesocosms the temperature was 5 °C higher, and in the remaining two mesocosms it was 10 °C higher than the control temperature. Any changes in temperature were controlled by a measurement system and temperature regulated by cooling or heating the water. To provide sunlight the mesocosms were placed outdoors. Temperature regulation system measured temperature in each mesocosm. Depending on the measured temperature, a control system started the heating or the cooling system.

Two lakes were studied, differing by the trophic status, located in the Great Mazurian Lakes (Poland) area:

- Lake Mikolajskie – eutrophic (surface area – 497.7 ha, max depth – 25.9 m, mean depth – 11.2 m)
- Lake Majcz Wielki – mesotrophic (surface area – 163.5 ha, max depth – 16.4 m, mean depth – 6.0 m).

Water sampled from the lakes was transported to the mesocosms and after setting the parameters, the system was left for one day to stabilize. The second day of the experiment was treated as “initial sample” and thereafter the analyses were done every second day during ten days. We measured primary production (14C-bicarbonate), phytoplankton composition, total number (TNB) and biomass of bacterioplankton (BB), bacterial secondary production (14C-thymidine), bacterial community composition (PCR-DGGE), bacterial liveliness (Live/Dead BacLight), DOC (Dissolved Organic Carbon) content, SUVA (Specific UV Absorbance), and nitrogen and phosphorus content.

The study revealed that regardless of the lake’s trophic condition rise in water temperature causes an increase of the primary production. However, the phenomenon occurred more dynamically in the mesotrophic lake, at the temperature rise to 30 °C. In consequence, high amount of labile organic matter was accumulated, which was displayed by the high DOC concentrations and the parallel low SUVA values, and by the high contribution of the organic forms of N and P on the 6th day of the experiment. At the temperature rise to 25 °C, the dynamics of the DOC changes was identical in both lake types.

In natural conditions (temp. 20 °C) the high activity of bacterioplankton in the mesotrophic lake provides for effective transfer of the matter to the higher trophic levels (proto- and zooplankton), which determines the high ecological stability of these ecosystems. Increase of the temperature may disturb this mechanism, as revealed in the experiment. In the mesotrophic lake TNB and BB dropped when temperature rose. The highest reduction was observed at the temperature rise to 25 °C. In the eutrophic lake at the water temperature of 20 and 30 °C in the following days of the experiment the DOC rose while TNB and BB decreased. At the temperature rise to 25 °C the system was more dynamic, yet the amount of organic matter constantly increased (high values of DOC, organic N and P).

We concluded that Mesotrophic lakes are therefore more vulnerable to temperature changes not only because organic matter increases which intensifies eutrophication but also because transformation processes by bacteria may be disturbed. In the mesotrophic lake at 20 °C organic matter was actively utilized in microbiological processes, as indicated by the clear growth of TNB and BB on the 4th day of the experiment, at the parallel decrease of the DOC and increase of the SUVA parameter value on the 6th day. In the first place bacteria utilized labile organic C, leaving in the water the DOC with a high number of aromatic rings in the molecule (higher SUVA).

In the global warming conditions the negative effect of such phenomenon may show through the reconstruction of the lake phytoecosystems structure, characterized by an increased occurrence of invasive, stenothermal and heavily toxic blue-green algae.

Keywords: temperature, biodegradation, organic matter, bacteria, DOC, lake

Bioeffects and biotransformation of selenate in Chlorella sorokiniana.

I. Garbayo, V. Ondruska, F. Moreno, T. García-Barrera, J.L. Gomez Ariza and C. Vílchez

Selenium is a trace element that acts either as an essential micro-nutrient or as a toxic element depending on its concentration in the medium. It is also of fundamental importance to human health; selenium bioeffects are mainly involved in immune function, reproduction, metal toxicity resistance and other biological functions in humans. Besides, selenium has been proved to be an effective antimicrobial agent when supplied in a suitable bioactive form. Microalgae can accumulate selenium. Selenium toxicity and accumulation in aquatic systems are difficult to study because of the complex aqueous chemistry of Se forms. In nature, selenium is present in three oxidation states (selenite (+IV), selenate (+VI) and elemental selenium (0)) over a range of natural water chemical conditions. Selenate is the dominant dissolved form, representing more than 67% of the total dissolved selenium concentration.

In the present work, Chlorella sorokiniana was chosen as a representative green microalga to evaluate the effect of selenium in selenate form on different growth parameters in order to produce Se-rich C. sorokiniana cultures:

- Growth, photosynthetic activity and selenium distribution with emphasis on the organic nature of the accumulated Se-metabolites, mainly selenomethionine.

In the eutrophic lake (Lake Majcz Wielki) the high activity of bacterioplankton in the mesotrophic lake provides for effective transfer of the matter to the higher trophic levels (proto- and zooplankton), which determines the high ecological stability of these ecosystems. Increase of the temperature may disturb this mechanism, as revealed in the experiment. In the mesotrophic lake at 20 °C organic matter increases which intensifies eutrophication but also because transformation processes by bacteria may be disturbed. In the mesotrophic lake at 20 °C organic matter was actively utilized in microbiological processes, as indicated by the clear growth of TNB and BB on the 4th day of the experiment, at the parallel decrease of the DOC and increase of the SUVA parameter value on the 6th day. In the first place bacteria utilized labile organic C, leaving in the water the DOC with a high number of aromatic rings in the molecule (higher SUVA).

In the global warming conditions the negative effect of such phenomenon may show through the reconstruction of the lake phytoecosystems structure, characterized by an increased occurrence of invasive, stenothermal and heavily toxic blue-green algae.

Keywords: temperature, biodegradation, organic matter, bacteria, DOC, lake

Acknowledgements: This work has been supported by the Ministerio de Educación y Cultura (Proyecto AGL2006-12741).
Biofertilization and phytostimulation in wheat by cyanobacteria

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Cyanobacteria are commonly used for phytostimulation and biofertilization of agriculture crops due to their nitrogen fixing ability however contribution by their phytohormones has been neglected. The study focuses on screening of rhizospheric and free living cyanobacteria for in vitro phytohormones production and growth stimulation in wheat. Selected isolates were shown to release as well as accumulate cytokinin and IAA by using UPLC coupled with MS/MS via electrospray interface. Maximum cytokinin and IAA concentration was 22.7 pmol mg\(^{-1}\) ch-at and 35.1 pmol mg\(^{-1}\) ch-at respectively in the culture medium of Chroococcidiopsis sp. Ck4 and Anabaena sp. Ck1. Growth of wheat inoculated with cyanobacterial strains was stimulated under axenic as well as field conditions. Seed germination, shoot length, tillering, number of lateral roots, spike length and grain weight were significantly enhanced in inoculated plants. Maximum increase in grain weight (43%) was demonstrated in wheat plants inoculated with Chroococcidiopsis sp. Ck4 under natural conditions. Positive linear correlation of cyanobacterial cytokinin with shoot length, tillering, spike length and grain weight was recorded. Cyanobacterial IAA on the other hand was positively correlated to the number of lateral roots. Endogenous phytohormones pool of the plant was enhanced significantly as a result of plant-cyanobacteria association in the rhizosphere. It was concluded that cyanobacteria used phytohormones as a major tool by to improve growth and yield in wheat.

Keywords: Cyanobacteria, cytokinins, IAA, UPLC-MS/MS, Phytostimulation, Biofertilization, Wheat

Bioluminescent monitoring of radiotoxicity in solutions of alpha-radiouclides

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Bioluminescent (BL) bacteria-based assay was adapted to monitor toxicity of alpha-radiouclide in solutions of low and mean activity. Chronic effects of alpha-radiouclides on BL bacteria Ph. Phosphoreum and their enzymes were studied. Radionuclides of different specific activity - \(^{241}\)Am(NO\(_3\))\(_3\) and UO\(_2\)(NO\(_3\))\(_2\) - were used as sources of alpha-radiation.

In Americium solutions, the BL activation predominated for short-term exposure (55 hr), and BL inhibition – for longer-term exposure to radiation (1). These effects were used to evaluate radiotoxicity of the solutions. The effects were shown to depend on radionuclide concentration, level of organization and integrity of the bioluminescent assay system. The BL activation was up to 480% for bacteria and up to 30% - for the enzymes. Accumulation of Americium in bacterial suspension was demonstrated. Damage of bacterial cell walls in Americium solutions was visualized by electronic microscopy images.

BL inhibition only was observed in the solutions of Uranium (2). Effect of Uranium was observed under higher concentrations (higher than 10\(^{-7}\)M), than that of Americium (down to 10\(^{-11}\)M). Influence of the radionuclides was compared to that of stable metals - Europium and Iron. The effects of Americium were attributed to its radioactive properties; however the effect of Uranium was due to its chemical properties.

The BL assay was used to monitor detoxification of alpha-radiouclides by Humic Substances (HS). Decrease of Americium effect on bacterial luminescence in the presence of HS was demonstrated: HS reduced BL activation and inhibition in the Americium solutions by 40 and 35 percent respectively. Decrease of bacteria damage and changes in Americium accumulation in HS solutions were found as well.

Keywords luminous bacteria, ionizing radiation, detoxification
Bioprospection and characterization of endophytic fungi from tropical mangrove forests

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Microorganisms from mangroves open up new areas of biotechnological exploitations, which drive the need to isolate and cultivate these organisms. The objective of this work was to evaluate genetic diversity of fungal endophytic community from mangrove forest in Bertioga, SP, Brazil. To accomplish this study, fungi were isolated from superficially disinfected tissues of three mangrove species (Rhizophora mangle, Avicenia schaueriana, Laguncularia racemosa). Fungal diversity was assessed by Amplified Ribosomal DNA Restriction Analysis (ARDRA) technique (Figure 1). In this study 1200 endophytic fungi were isolated from branches and leaves of the mangrove species. It was observed at least 7 different morphological groups. Both leaves and branches presented all these groups. However, it was observed difference when the isolation frequency from leaves and branches were compared, that is, branches had higher colonization density than leaves. ARDRA analysis showed that 80 fungi isolated were separated in 21 different haplotypes (Figure 1). The comparative analysis of the different haplotypes suggested that mangrove associated fungal community from Bertioga is compounded from the haplotypes that occur in all treatments (place, species and tissue). As for the biotechnological potential some endophytic isolated were tested for production of antibiotic against Staphylococcus aureus, Escherichia coli and Xanthomonas campestris citri (Figure 2). The ITS1-5.8S-ITS2 rDNA region sequence revealed that the endophytic fungal community isolated from mangrove species include the following genera: Gibberella, Colletotrichum, Hypocrea, Phomopsis, Fusarium, Xylaria, Diaporthe, Alternaria, Gelasinospora, Trichoderma, Cylindrocladium, Cytospora, Phaeoasphalticopsis, Colletotrichum, Botryosphaeria, Guignardia, Glomerella, Dokitria, Arthothelium, Penicillum, Neurospora, Coprinellas, Glomus, Coniothyrium, Pleosporales, Leptosphaeria, Lastudiplodia, Phaeoseptoria, Amorosia, Nodulisporium, Phaeoramularia, Botryosphaeria, Nigrospora, Pseudalesscheria, Paraconia, Microthia, Neofuscosum, Pestalotiopsis, Crysophycrea, Epicoccum, Valsa, Sordariomycetes, Dothideomycetes, Massarinia, Pichia, Basidiomycete, Cladosporium. The study of the fungal community is now focused on investigating of the chemical structure of antibiotics produced by these isolated.

Keywords: ARDRA; antibiotic; fungal diversity; Rhizophora; Avicenia; Laguncularia

Figure 1. Restriction profile (ARDRA) of some haplotype found in the endophytic fungal community isolated from mangrove species.

Figure 2. Antagonistic activities using concentrated extract of endophytic fungi isolated from mangrove against Escherichia coli. Inhibition halo indicated by white arrows.

Can microbial decomposers of plant litter be used as bioindicators of anthropogenic stress in streams?

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Human activities have greatly affected freshwater ecosystems worldwide, threatening biodiversity and putting at risk ecosystem functions and services. Therefore, there is a need of identifying new and effective bioindicators of anthropogenic stress to protect against continued degradation of freshwaters. In streams, microbial decomposers, namely bacteria and fungi, are recognized to play an important role in organic matter turnover and improve plant-litter palatability for invertebrate consumption. In this study, we examined the impacts of eutrophication and heavy metals on microbial communities associated with decomposing plant litter in streams. The exposure to increased concentrations of inorganic nutrients (nitrogen and phosphorus) or heavy metals (copper and zinc) significantly reduced fungal diversity and reproduction, revealed by the number and morphology of released conidia from decomposing leaves. Fungal and bacterial communities assessed from DNA fingerprints (PCR-DGGE analyses) were structured by the level of eutrophication or heavy metals in the stream water, as shown by multivariate analyses. These results suggest that aquatic microbial decomposers of leaf litter respond to water chemistry and may potentially be used as bioindicators of stream ecosystem condition.

Keywords: freshwaters; biodiversity; bioindicators; aquatic microbial decomposers; eutrophication; heavy metals

Acknowledgement. The Portuguese Foundation for Science and Technology supported S. Duarte (SFRH/BPD/47574/2008)
Characterization of haloophilic microorganisms from the Brazilian Northeast saline soil

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Salt tolerance and osmotic regulation mechanisms from several organisms are still the subject of many studies. Their understanding is interesting for academic and biotechnological purposes, especially because of the worldwide increase of saline soils. Therefore, the characterization of haloophilic organisms is important to unravel the salt tolerance mechanisms. In this work we present the initial characterization of two microorganisms isolated from soil of salt evaporation ponds in Mossoró, Rio Grande do Norte, Brazil. The isolates were able to grow on rich media with 2.56 M NaCl (15% w/v) but unable to grow on 3.42 M NaCl (20% w/v), and grew very poorly or didn’t grow (depending on the experiment) in media without salt, which stresses the importance of high salinity for their metabolism. However, these isolates could not grow on rich media with 15% w/v KCl or 15% w/v KCl plus 2% w/v glucose or 2% w/v sucrose, indicating that the KCl was somewhat toxic. These results suggest the existence of a Na+/K+ antiporter whose activity is important for salt tolerance. This hypothesis will be tested with the use of pump inhibitors. The isolates were considered bacteria because there was amplification of a 1.6 Kb DNA band from their total DNA when bacterial 16S ribosomal gene primers were used, whereas no amplification was detected with Archaeal 16S ribosomal gene primers. These amplified DNAs will be sequenced to allow primary identification. Another ongoing approach to identify the tolerance mechanisms is the screening of genomic sequences that would confer higher salt tolerance on yeast. The genomic library on yeast expression vector is currently being constructed.

Keywords: salt tolerance; bacterial characterization

Supported by FAPEMIG (CBB APQ-2337-3.12/07)

Chemically treated fig tree leaves as low-cost biosorbent for removal heavy metal lead from aqueous solutions

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Contamination of the environment by lead is recognized throughout the world as one of the major environmental problems. This study was focused on the application of some low-cost biosorbents for removal of heavy metal lead from aqueous solutions. After the screening some locally available agricultural waste materials containing rice husk, tea waste, orange peel, tamarind seeds, tea leaves, fig tree leaves and tamarind fruit shell as potential low-cost biosorbents in a metal synthetic solution containing 85.5 mg/l Pb2+ ion and at varied pH, the best adsorbent was selected. The metal binding capacity differed for the applied adsorbents ranging from 10.95 to 37.55 mg/g dry wt. Some of the biosorbents showed higher rate of Pb2+ adsorption but the biomass prepared from fig leaves was more effective for removal of Pb2+ ions in compared with other biosorbents. As a result 88% of Pb2+ ions removed using fig leaves. It was observed that the adsorption capacity of fig leaves increased (41.95 mg/g dry wt) after treated with nitric acid (65% w/w) so the other experiments were carried out with modified fig leaves. Batch experiments were conducted to determine the factors affecting adsorption of Pb2+ ions such as adsorbent dosage and initial lead concentration. pH dependence of metal uptake and kinetics of metal adsorption were investigated. The adsorption of lead was found to be maximal at pH in the range of 4-4.5. The experimental studies showed the time about 2 hours is sufficient for removal of 97.55% of Pb2+ ions from solutions with concentration 85.5 mg/l Pb2+ ion by chemically treated fig leaves. The Langmuir and Freundlich models were used to analyze the experimental data.

Keywords: Heavy metals, Low-cost agricultural products, Biosorption, Modified fig leaves, Lead
Chitinolytic Bacteria Isolated from Chili Rhizosphere: Chitinase Characterization and Application As Biocontrol for whitefly (Bemisia tabaci Genn.)

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Chitin which is a common constituent of insects exoskeleton could be hydrolyzed by chitinase. The research was conducted to select rhizobacteria isolated from the rhizosphere of chili pepper which could produce chitinase and examine their chitinase activity in degrading chitin of whitefly, Bemisia tabaci Genn. (Hemiptera: Aleyrondidae). The whitefly is recognized as an important pest on many crops. It attacks more than 500 species of plants from 63 plant families including chili pepper. A total of 25 isolates rhizobacteria formed a clear zone when they are grown on solid chitin media. Two of isolates had the highest chitinolytic index, i.e. I.5 and I.21. Based on sequencing the 16S rRNA gene, the isolate I.5 and I.21 was identified as Bacillus sp. and Bacillus cereus, respectively. The highest chitinolytic index and specific activity of strain I.5 was 0.94 and 0.11 U/mg proteins, respectively. Maximum production of I.5 chitinase was occurred after 36 hours cultivation under 30°C and pH 7.0. The highest chitinolytic index and specific activity of strain I.21 was 0.75 and 0.114 U/mg proteins, respectively. Maximum production of I.21 chitinase was occured after 36 hours cultivation under 55°C and pH 7.0. The cell culture and the enzyme of two isolates were tested on B. tabaci and the result in microscopic observation was compared to control by using sterile water. Hydrolytic analysis showed that enzyme of the I.21 isolate could be degraded the chitin of B. tabaci exoskeleton was better than I.5 isolate. Chitinase produced by Bacillus cereus strain I.21 is potential as biocontrol agents for B. tabaci.

Keywords: Chitinolytic: rhizobacteria; chitinase; biocontrol; Bemisia tabaci Genn.

Chromobacterium sp. from the tropics: detection and diversity of phytase activity

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Phytases are a group of enzymes that catalyze phytic acid hydrolysis with release of P, and are found in many organisms including bacteria. Despite the expanding use of phytase for biotechnological applications, information on phytase-producing bacteria is limited. The ability of Brazilian Chromobacterium sp. isolates to produce phytase was evaluated. Altogether, 125 candidate bacteria isolated from Brazilian Savannah (Cerrado), Atlantic Rain Forest and Amazon Rain Forest were tested. Qualitative assays revealed 115 phytase-producing isolates. The phytase production by Chromobacterium isolates was also confirmed by quantitative tests through detection of free P. Although the genome of a Chromobacterium violaceum type-strain has been sequenced, this is the first report revealing the genus Chromobacterium as phytase producer. The results presented in this paper suggest great diversity of phytase production among the Brazilian isolates and indicate a potential use of them for commercial prospects.

Keywords: Chromobacterium sp.; phytase; phytate; diversity; tropical bacteria

Supported by FAPEMIG and CNPq
Comparative analysis of three molecular techniques used in the biodiversity study of a thermomineral spring cyanobacterial mat

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Naturally occurring cyanobacterial mats were highly investigated in the last decades due to their resistance and productivity, especially in extreme environments, which are completely inaccessible to eukaryotic organisms. The structure of bacterial communities in the environment has been investigated by culture-dependent methods for many years. However, since in ecological studies of cyanobacteria it is essential to be able to distinguish closely related organisms and because it is difficult to culture most bacteria from environmental samples, evaluation of changes in the structure of bacterial communities using only culturing methods is inadequate. A recent approach in the study of cyanobacterial communities is represented by the use of molecular techniques, which approach leading to the discovery of unique and previously unrecognized microorganisms. The majority of these studies are using partial sequence data from the rrs operon as markers (the 16S rRNA gene and the Internal Transcribed Spacer-ITS). The degree of sequence heterogeneity, as well as a considerable number of published sequences, make the rRNA-ITS fragment very suitable for high resolution analysis of cyanobacteria. This study discusses the suitability of Denaturing Gradient Gel Electrophoresis (DGGE) technique in the study of a cyanobacterial mat associated to a thermomineral spring from the Western Plain of Romania, alongside with two additional techniques: Automated Ribosomal Intergenic Spacer Analysis (ARISA) and Amplified Ribosomal DNA Restriction Analysis (ARDRA).

For DGGE, the 16S rDNA variable regions V3 and V4 were amplified, the resulting 8 bands excised and sequenced. Due to overlapping peaks obtained after sequencing, a clone library was constructed for every individual band. 40 clones (5 clones for each of the 8 bands) were re-sequenced by DGGE and sequenced. The electrophoretic profile and the DNA sequences obtained revealed a variety of disadvantages regarding this technique which can easily lead to improper interpretation of the results: multiple melting domains in the same sequence, multiple rrs operons in the same genome and unspecific hybridization among DNA fragments from different species. To see if the results obtained in DGGE are caused by certain PCR amplification bias, the species abundance and their identification was done using both the ARISA and ARDRA methods.

As stated in other ecological studies, ARISA is a rapid and effective quantitative method for assessing microbial community variety. The ITS fragment was amplified by PCR and the amplification products were discriminated by capillary electrophoresis using the ABI Prism 310 genetic analyzer. A total of 13 different ITS fragments were obtained, corresponding in theory to 13 different cyanobacterial species. This step is very important for further ARDRA analysis because it offers an overall view of the number of species in the mat, so that the appropriate number of clones will be selected in order to gain optimal results after restriction profiling.

For ARDRA, a clone library for the 16S rDNA-ITS fragments was constructed. Plasmids from 46 colonies which tested positive after the blue-white selection were isolated and used as template for the re-amplification of the 16S rDNA-ITS fragments. The amplification products were digested with TaqI restriction endonuclease and migrated on an agarose gel. Estimating the size of the fragments obtained after restriction, a cluster analysis was performed for the restriction profiles. The different 16S rDNA-ITS fragments were sequenced and the sequences obtained compared to the ones existing in the international nucleotide databases using blastn algorithm (NCBI) for species identification. The investigated mat presented 8 cyanobacterial species, Phormidium and Leptolyngbya being dominant.

This study demonstrates that DGGE, even though has many advantages, can lead to an incorrect and overestimated species diversity in specific cyanobacterial mats with closely related species due to multiple rrs operons, multiple DNA melting domains and unspecific hybridization. Our results suggested that, despite using more variable regions in the 16S rRNA gene and very specific primers as indicated by other authors, in certain conditions a single DGGE band can represent a mixture of different species or that bands which migrated at different positions in the gel represent the same DNA fragment with multiple melting domains. Using multiple molecular techniques alongside with DGGE, such as ARISA and ARDRA, can improve the microbial biodiversity studies, thus providing optimal results.

Keywords cyanobacterial mat, 16S rRNA, DGGE, ARDRA, ARISA

Comparison of Antibiotic Resistant Staphylococci on Hands of College-aged and Pre-school Aged Students

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The increased resistance of bacteria like Staphylococcus, a typically harmless bacteria of our natural flora, to antibiotics has become a concern in the general community with particular regard to protecting our children. Stress to the natural flora over time can increases the resistance of the natural flora. Stress can cause favorable gene mutations to be selected for and increase gene transfer between bacteria. This study was conducted to determine if there is a difference in antibiotic resistance of the natural flora on the hands of College-aged students and Daycare-aged students.

To ascertain the potential role age plays in the resistance of our natural flora to antibiotics, samples of hand bacteria were collected from both daycare age students of 3-5 and college-age students of 18-22 using a sterile sponge and 20mL of sterile saline solution. Twenty μL of each sample were incubated for 24 hr in solutions of the 10 different antibiotics at their minimum inhibitory concentration (MIC). The antibiotics included linezolid, chloramphenicol, ampicillin, streptomycin, nisin, vancomycin, ceftriaxone, ciprofloxacin, oxacillin and rifampicin. After 24hr that samples were plated on mannitol salt agar to select for Staphylococcus. Where growth was seen resistance was recorded.

Average Antibiotic Resistance per-Person Percent Antibiotic Resistance with in Group

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Child</th>
<th>Adult</th>
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</thead>
<tbody>
<tr>
<td>Linezolid</td>
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<td>Rifampicin</td>
<td>3.28</td>
<td>0.01</td>
</tr>
</tbody>
</table>

These results show significantly that in this test population college-aged students natural flora are resistant to more antibiotics than daycare-aged students natural flora. However some have high resistance in both test populations. It is possible that exposure to the environment over time does increase the antibiotic resistance of natural flora on human skin.
Comparison of antimicrobial activity in *Sinularia compressa* from two different ecological conditions of the Persian Gulf

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Patterns of intraspecific variation in composition, concentration and bioactivity of secondary metabolites among geographic regions or habitats have been well documented for terrestrial plants. In the marine environment, however, chemical variation has received less attention.

In this study, samples of *Sinularia compressa* were collected from two different zones: air-exposed from Larak Island and non air-exposed from Hengam Island using SCUBA equipments in 8-10 m depth. Extraction and assay protocols were developed to identify antimicrobial activity in some fractions of extracts. Detection was determined by disc assay method with antibiotic as control, for each sample, using two gram positive bacteria, (*Staphylococcus aureus* and *Bacillus* sp.) and four gram negative bacteria, (*Pseudomonas* sp., *Klebsiella* sp., *Salmonella* sp. and *Shigella* sp.). TLC, ninhydrin and vanillin/sulfuric acid reagents and GC-MS were used to isolate, identify and characterize metabolites in fractions. Also production of biofilm by *Pseudomonas* sp., quorum sensing inhibition test by *Chromobactrium violaceum* and brine shrimp toxicity test were used to compare the extracts. The results demonstrated that extracts of *Sinularia compressa* show different characteristics due to their ecological habitats. For example some fractions of *S. compressa* extract from Larak Island, such as acetone and water fractions exhibited appreciable antimicrobial activity on some bacteria, while these fractions of *S. compressa* extract from Hengam Island had little or no antimicrobial activity. The outstanding feature of these different effects is the qualitative variation in the chemistry of colonies from different habitats, which appears to be genetically fixed, raising the question of what selective agents maintain the variation in chemistry and bioactivity among different habitats.

**Keywords** antimicrobial activity; *Sinularia compressa*; ecological conditions; Persian Gulf

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Comparison of experimental methods for determination of toxicity and biodegradability of xenobiotic compounds

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Chlorophenols are a group of organic pollutants present in the environment as a result of several industrial (like bleaching of pulp with chlorine) and man-made activities, such as waste incineration and uncontrolled use of wood preservatives, etc. They have obtained notoriety as hazardous substances, because most of them are toxic, and present long persistence in the environment. Other important environmental problem is the pollution of soils and aquatic systems by chemicals used in agriculture, such as herbicides. Those compounds, with widespread use all over the world, low solubility in water, strong persistence and possible toxic intermediates, are clearly bound to seriously contaminate soil and aquatic environments.

In our work 4-chlorophenol (4CP), 4-chlorocatechol (4CC), pentachlorophenol (PCP), 2,4-dichlorophenol (2,4DCP), 3,5-dichlorophenol (3,5DCP) and 2,4,6-trichlorophenol (TCP) are studied as representative of xenobiotics occurring in many industrial wastewaters whereas atrazine, alachlor, diuron, MCPA and 2,4-D are analyzed as representative of pesticides. As they constitute a threat to human health and produce a public concern, several of those compounds are included in the list of priority hazardous substances of the European Union (Directive 2008/105/EC).

The aim of this work is to compare different microbiological methods for determination of toxicity and biodegradability of toxic compounds. Toxicity was estimated in terms of EC50 and evaluated by two of the most utilized methods: a simple respirometric procedure set up on the basis of OECD Method 209 and by the Microtox® bioassay. For determination of ready biodegradability an easier and faster alternative to the OECD Method 301 is proposed. When this test was negative, Zahn-Wellens test (OECD 302B) was performed in order to evaluate inherent biodegradability. Both tests were performed with an initial concentration bellow the corresponding EC50 value.

The activated sludge was obtained from a municipal treatment plant. Chlorinated compounds and pesticides were analyzed by HPLC with UV detection. Total organic carbon (TOC) was analyzed by direct injection of the filtered samples into a Shimadzu TOC-VCSH analyzer.

Comparison of EC50 data obtained with the two methods showed that in both cases chlorinated compounds (respirometric EC50 = 14-117, Microtox EC50 = 0,06-12) are more toxic than the pesticides (respirometric EC50 = 139-250, Microtox EC50 = 11-292) investigated and alachlor had a very low toxicity value. Moreover, the Microtox EC50 values were generally much lower than the respirometric ones.

The biodegradability test proposed indicated that only 4CP and 4CC among chlorinated compounds are readily biodegradable, whereas Zahn-Wellens test showed a complete biodegradation for TCP and 2,4 DCP. 3,5 DCP appears to be the most recalcitrant phenolic compound, with a extent of degradation of only 15% after 28 days. Among pesticides, only biodegradation of alachlor was achieved to an appreciable extent (80%) using the Zahn-Wellens test. Although chlorinated compounds, in general, showed higher toxicity than pesticides, surprisingly they appeared to be more biodegradable for a partially adapted microbial community.

In conclusion, the comparison of these toxicity methods shows that both can be usefullly applied for toxicity detection in wastewater treatment plants. Respirometry is a specific method since the toxicity effects are evaluated directly on the plant activated sludge. Nevertheless, as biodegradability data shows, the evaluation of toxicity values in not enough to classify a wastewater as easily biodegradable.

**Keywords** chlorinated compounds, pesticides, toxicity, biodegradability, respirometry measurements, Microtox®, test Zahn - Wellens

**Acknowledgements**

This work was financially supported by Comunidad de Madrid - Universidad Autónoma de Madrid through the project CCG08-UAM/AMB-4436 and by the Spanish Ministerio de Ciencia e Innovación through the project CMT2007-60959.
Correlation between PRTF1-F2 and macrolide resistance in *Streptococcus pyogenes*

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Eighty-eight strains of *Streptococcus pyogenes*, isolated from tonsillar infections in children and young adults during a national multi-centric study in various regions of Italy, underwent genotypic-phenotypic investigations to establish the genetic determinants of erythromycin-resistance and the possible correlation with the virulence factors *prt*F1-F2.

The most frequent resistance phenotype to erythromycin was cMLSb (40.91%), followed by iMLSb (36.36%) with the resistance determinant *erm*(B) more prevalent with respect to *erm*(A) and *mef*(A), and well as with respect to those of *erm*(B) was found exclusively or simultaneously in the same strain. The phenotype M (22.72%) was, as was predictable, characterized in 100% of the cases by the presence of *mef*(A), and in almost all cases (95%) its presence was exclusive with respect to *erm*(B) and/or *erm*(A).

The genetic determinants *prt*F1 and *prt*F2 encoding the FBP, adhesion proteins were present in a high percentage and comparable to both the sensitive GAS, and to the GAS resistant to erythromycin. In particular, *prtf*F1 was found in 100% of the strains with phenotype M. Moreover, Multilocus Sequence Typing, carried out on a pool of representative strains allowed the examination of the genetic background of the strains and discovered four new ST461, 462, 463, 464, of which two from new variants of the locus of the housekeeping genes *gtr* and *muts*.

**Keywords:** *Streptococcus pyogenes*, virulence factors, genetic determinants

Correlation between growth rate and donor/recipient ability in natural *E. coli* strains

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In this work we intend to measure growth rate values for *Escherichia coli* strains and relate these values with information regarding these strains behavior in plasmid conjugation.

*E. coli* strains used in this study were randomly chosen from a collection of natural isolates and used in a study for the determination of conjugation rates among themselves with plasmid R1. Donors and recipients were distinguished by isolating spontaneous antibiotic resistant mutants before the introduction of plasmid R1. Donors are resistant to nalidixic acid and mecillinam and the presence of plasmid R1 confers additional resistance to ampicillin, chloramphenicol and kanamycin.

Maximum growth rate values for donor strains with and without plasmid R1 are expected to vary accordingly to the plasmid cost. We anticipate a relation between growth rate and the strains donor/receptor abilities, which were already obtained in a previous study and found to reveal great diversity. We expect that the worst donors will have a lower growth rate because they are not spending much effort in passing the plasmid to other strains. The worst receptors will probably also have a lower cost because they might have lost another plasmid due to incompatibility with R1 during its acquisition.

**Keywords:** growth rate, conjugation rate, plasmid cost, plasmid R1
Decolorization of a real textile wastewater by marine Aspergillus niger

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Wastewater originating from the textile-processing industries is a complex mixture of potentially polluting substances consisting of textile dyes, heavy metals associated with dyes and the other auxiliaries used during dyeing process (Sharma et al., 2004). It is estimated that from 1 to 15% of the dye is lost during dyeing section of a textile industry process and is released in wastewaters (Baraka et al., 2009). And most of the azo dyes, which are released into the environment, originate from the textile industry and the dyestuff manufacturing industry. In this study a real wastewater from Barka Textile Company in Tehran was decolorized by a fungal biomass. A strain of Aspergillus niger that was isolated from marine showed a high capacity for rapid decolorization of textile dyes solutions and textile effluent. The optimization experiments demonstrated that decolorization was as much as 96% when sucrose was as carbon source at concentration 5g/liter of effluent. Addition of various nitrogen sources showed the best source is NH4Cl but different concentrations of NH4Cl had not significantly effect on decolorization. The reduction of dyes was maximal at original pH of effluent (7.5-9) and at 30 °C. Under optimal conditions, also 97% of dyes were adsorbed from textile effluent in 22 hours by approximately 1g dry weight of cells per liter of effluent. The kinetics of decolorization in batch culture showed that more than 90% of dyes in effluent removed in the first 10 hours after contact. Dyes strongly bound to the fungal biomass required sonication in dH2O, Triton X-100 and extraction with methanol for their removal. The results of cell fractionation showed decolorization by fungus were via surface and internal cell adsorption. Although, surface adsorption was significant (65.14% of decolorization).

Keywords: Fungal biomass, Textile wastewater, Decolorization, Azo dyes, Decolorization kinetics

References
Denitrifiers community abundance, structure and function associated with salt marshes sediments.

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Denitrification in eutrophic coastal systems influences the nitrogen budget and may result in increased fluxes of nitrous oxide (N2O), a potent greenhouse gas that also contributes to the destruction of the ozone layer. Denitrification can be physically and biochemically influenced, since sediment characteristics and organic carbon availability may be affected by the presence of plants. In this study, denitrifier communities abundance structure and activity were studied in salt marshes colonized (rhizosystems) and un-colonized sediments with different degrees of contamination: one in the Cávado estuary (NW Portugal), and two (Lisnave and Comporta) in the Sado estuary (SW Portugal).

Rhizosystems from Halimiones portulacoides meadows and un-colonized sediments from the same locations were sampled seasonally during one year. Denitrification and nitrous oxide (N2O) production rates were measured in sediment slurries experiments using the acetylene block technique. Total number of bacteria was estimated by means of direct microscopic count of DAPI-stained cells. The diversity of genotypes of nitrate (narG), nitrite (nirS and nirK) and nitrous oxide reductase (nosZ) genes were evaluated by DGGE and cloning and quantitative analysis of nirS and nirK was performed by real time PCR.

Denitrification potential presented a strong temporal variation, with higher rates during the summer and fall. Rates of N2O production were found to be higher in sediments colonized with plants than in un-colonized sediments. This fact can reflect differences in the abundance and structure of the microbial community. In the majority of the samples bacteria were more abundant in rhizosystems than in un-colonized sediments. Moreover, cluster analysis of DGGE profiles showed differences in denitrifier assemblages composition; while in Cávado estuarine seasonal differences in denitrifying community structure reflected seasonality, in Sado estuary differences between rhizosystems and un-colonized sediments surpassed seasonal differences in denitrifiers community.

These results suggest that denitrifier communities are adapted to distinct environmental pressures and that community structure alone cannot explain all the differences in denitrification rates. Moreover, denitrifier communities in rhizosystem can have an important contribution to the greenhouse effect through N2O emissions. Since salt-marshes can colonize large areas in temperate estuaries, the dynamic of denitrification pathway in these sediments should not be disregarded.

This work was partially funded by Fundação para a Ciência e Tecnologia (FCT), Portugal, through project POCI/CTA/48386/2002.

Keywords salt marshes; denitrification; rhizosystems

Desulfurization of Crude Oil by Rhodococcus erythropolis cells

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The refinery industries are facing nowadays the challenge of simultaneously keeping production yields high while limiting the release of toxic elements, such as sulphur dioxides, into the environment. Scientists all over the world have been targeting at the discovery of new economic and efficient methods for desulfurization of recalcitrant sulfur-containing organic compounds. Biodesulfurization (BDS) is a process that uses microorganisms such as Rhodococcus erythropolis, as the catalyst for fuel desulfurization. R. erythropolis cells are gram-positive and are known to be resistant to a large number of recalcitrant compounds. These cells have the ability to degrade hydrophobic natural compounds and xenobiotics, including dibenzothiophenes (DBTs) [1] and therefore are important tools for successful bioconversions and bioremediation. DBT is one of the most abundant sulfur components in the crude and has been widely used as the model compound for crude oil desulfurization research studies. The feasibility of using two different strains of R. erythropolis for the desulfurization of crude was investigated. The experiments showed that both R. erythropolis DCL14 and R. erythropolis IGTS8 were able to use DBT as sole sulfur source. A concentration of 0.1mM of DBT was used and HPLC chromatograms showed that both strains were able to metabolize rapidly the compound. For R. erythropolis IGTS8 cells a comparison between resting and growing cells was also performed. R. erythropolis IGTS8 resting cells were able to completely metabolize 0.1mM DBT in less than two hours whilst growing cells took up to 15 hours to metabolize the same DBT concentration.

Keywords: Desulfurization, Rhodococcus, Crude oil

References:

Acknowledgements: This work was performed under the MIT-Portugal program. I.F.F acknowledges Fundação para a Ciência e Tecnologia for the BD 38941/2007 PhD fellowship.
Detection of D/N functional genes during a biotreatment of mixed olive oil and winery wastewaters

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Several yeast may cause human and animal opportunistic infections, like the genera Cryptococcus and Candida. Cryptococcosis is a systemic fungi infection that may be acquired by inhalation of airborne particles contaminated with capsulated yeasts of the Cryptococcus genera. This disease has worldwide distribution. The prevalence of Portuguese wineries and olive oil mills are often located in the vicinity of each other which makes possible to share human and animal cryptococcosis has increased in the past 20 years for several reasons, including AIDS and FIV, wastewaters treatment plants (WWTP) to treat both effluents, before being discharged into municipal collectors and/or transport to one WWTP. This economy of resources is very important since Portuguese wine and olive oil is produced by small or medium companies.

Biotreatment monitoring is the unavoidable factor to characterize, control and improve the process, which will lead to new strategies for better treatments efficiency.

In order to optimize biotreatments for mixtures of the two referred effluents, a study was carried out in a jet-loop aerobic reactor, adding a winery effluent during the final phase of an olive oil wastewater biotreatment. Online determination of the main physical/chemical parameters (pH, NO3, temperature, and 50%O2) was performed by sensors connected to new software developed to this aim associated with an acquisition data system plate (National Instruments). Off-line determination of COD, VSS and TSS was done following standard methods, and off-line NH4-N measurements were performed with a probe (Crison) selective for NH4-N ion.

Bioreactor started-up under a batch phase, followed by a continuous regimen, testing two hydraulic retention times (HRT) of 6.0 and 4.5 d, corresponding to feeding flow rates of 3.75 L/d and 5.0 L/d, respectively. Winery effluent was fed at a HRT of 6.0 d (3.75 L/d). COD olive oil wastewater removal rates reached more than 65%, at a loading charge of 16.1 g COD.l-1.d-1 with a HRT of 4.5 d.

Effluent biotreatments efficiency also depends upon biological nitrogen removal carried out by denitrifying/nitrifying (DN) microorganisms. To detect the functional genes involved in the nitrogen cycle (N/D processes), samples were collected at selected phases of the treatment, and total DNA was extracted, and was amplified by PCR with specific primers for nifH, napH, nirK, nitR, nosZ, amoA and norB genes.

The relevance of the molecular methods is nowadays recognized as the most important set of tools to identify unknown microbiota but this methodology does not provide cultures of a desired microorganism. To access and identify cultivable members of the consortia, some samples were also subjected to a cultivation, isolation and tipification experimental procedure using classical microbiological techniques. For sequencing analysis, 16S rDNA of isolates was amplified by PCR with universal primers for Eubacteria. To determine the phylogenetic affiliation, similarity search was performed using the BLAST program. The nucleotide sequences were aligned by the CLUSTAL program and the phylogenetic tree was constructed. Eleven sequences of isolates affiliated with phylum Firmicutes, one with Actinobacteria, and one with sub-class alpha-Proteobacteria.

Acknowledgments Funding from FCT Project MOTIVE (PPCDT/AMB/56616/2004). Authors wish to thank J. C. Duarte from LINSEG (Unidade de Bioenergia) for availability of jet-loop reactor.

Keywords olive oil wastewater; winery effluent; microbial consortia; jet-loop reactor; D/N functional genes

Detection of potentially pathogenic yeast on environmental sources in Portugal

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Several yeast may cause human and animal opportunistic infections, like the genera Cryptococcus and Candida. Cryptococcosis is a systemic fungi infection that may be acquired by inhalation of airborne particles contaminated with capulated yeasts of the Cryptococcus genera. This disease has worldwide distribution. The prevalence of human and animal cryptococcosis has increased in the past 20 years for several reasons, including AIDS and FIV, expanded use of immunosuppressive drugs, elderly population and organ transplantations. Generally this agent is found in immunocompromised hosts, but it can cause also disease in healthy individuals. Large populations of roosting birds may represent a risk of infection to people living nearby. The most serious health risk arise from birds’ faeces, in which the Cryptococcus can grow due to the nutrient-rich accumulations of pigeon’s droppings (creatinine), feathers and debris under a roost - particularly if roost has been active for years. In natural conditions, this Basideomycete can also be associated with several different trees, such as eucalyptus trees. Infection is typically caused by inhalation of air-borne propagules and skin injuries, which can lead to hemolympathic and pulmonary tissues dissemination. Since the initial reports in Portugal, researchers have greatly uneasiness from a harmless colonization of the pigeons. Avian faeces droppings and the incidence of certain species of Eucalyptus trees, as original sources of C. neoformans on the public urban areas, may increase the exposure risk in immunocompromised patients. Despite the lack of information concerning to the occurrence of this systemic mycosis, about 2 to 10% of AIDS patients died with it. Candida genera are also very frequently found in environmental sources, although most of the human infections are autogenic. Candida species account for up to 50% of deep candidiasis cases, yet little attention has been paid to the virulence attributes of these fungi. C. albicans can cause serious disease in HIV-seropositive patients. In this context the aim of this work is to provide information concerning to environmental sources of Cryptococcus and Candida and its potential health risk for some human groups, namely pigeon breeders and immunocompromised patients (risk groups).

Keywords Cryptococcus, pigeon’s faeces, eucalyptus, Candida
### Detoxification of Olive Mill Wastewaters Using a Packed-Bed Batch Reactor

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Olive oil production is a traditional agricultural industry in Mediterranean countries and Portugal is one of the ten major producers. This industry generates an effluent, olive mill wastewater (OMW), which does not undergo any treatment and, usually, is stored in evaporation lagoons or spread on the land. Disposal of olive oil mill wastewaters is a serious environmental problem due to its high organic loading, presence of polyphenols and tannins, high content in suspended solids and acidity, which contributes to its ecotoxicity.

In this work, a biological treatment system: a packed-bed batch reactor was applied to a Portuguese OMW using its autochthon microbial population as inoculum. Thus, the biodegradation potential of OMW’ microorganisms naturally present in these wastewaters was assessed monitoring several physico-chemical parameters along the process. Ecotoxicity tests (Pseudomonas putida growth inhibition test and Vibrio fischeri growth inhibition test) were carried out to follow the detoxification capacity of the system as well as its potential to be used in the treatment of this type of agroindustrial effluent.

In this aerobic treatment, an active microbial community with high degradation ability for the OMW organic load was detected, accounting for 80%, 71% and 63% removal of COD, TSS and phenols, respectively. In addition, a significant decrease in the chronic toxicity of the treated OMW to both bacteria, *V. fischeri* (62.8%) and *P. putida* (64.3%), was also observed after 140 days of treatment, highlighting the detoxification potential of the system studied.

**Keywords:** OMW; detoxification; packed-bed batch reactor; ecotoxicity tests.

### Dissipatrophic bacteria, which develop in community with xylolytic fungi in the ultrafresh conditions.

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Wood decomposition is one of scale processes in carbon circulation in boreal zone of Russia. The basic destructors of wood are xylolytic fungi which activity leads to formation of dystrophic waters in forest and marsh ecosystems. Bacteria are not capable to destruct chemically inert lignin, but they are capable to use low-molecular products of lignin hydrolysis by fungi. Thus, bacteria and fungi form mico-bacterial community which is characterized by close interrelation of its components (bacteria and fungi). Acidic dystrophic waters are formed during an initial stage of wood decomposition. Studying of bacterial community from such environment is of great value for water use.

The purpose of the present work is:

1) to study a specific variety of bacteria developing in ultrafresh acidic waters, generated by xylolytic fungi in the course of wood decomposition and

2) to establish a functional role of these bacteria in conditions of laboratory model.

Different xylolytic fungi such as *Aspergillus ustus*, *Penicillium decumbens*, *Penicillium sp.*, *Trichoderma harzianum*, *Cladosporium sp.* were revealed in investigated community. Oligotrophic bacteria with characteristic morphology belonging to genera: *Caulobacter sp.*, *Prosthecobacter sp.*, *Hyphomicrobium sp.* were found out in wash waters of laboratory model at pH 4.3 and electroconductivity 140 μS.

A number of pure cultures of acidophilic dissipatrophic bacteria was isolated. These bacteria are indicator strains for the given stage of wood decomposition. Eco-physiological characteristics of nine of them are under detailed study at present.

On the basis of phones and phylogenetic analysis of 16S rRNA gene sequences isolated cultures were identified as genera: *Xanthobacter* (strain Z-0055, which is described as *X. xylophilus* sp. nov.), *Methyllobacterium* sp. (strain Z-0033), *Hyphomicrobium* sp. (strains Z-0045), *Microccocus* sp. (strain Z-0066), *Alphap* sp. (strain Z-0043), *Spirosoma* sp. (strain Z-0088), *Acynobacter* (strain Z-0056, which is a new species of this genus). Also two acidophilic representatives of order Planctomycetes (strains Z-0077 and Z-0078) were isolated. The investigated group of bacteria is characterised by following features: acidophily (pHopt for growth within 5.0-6.5); ability to grow only at low concentration of NaCl in medium (not above 0.25%, with the exception of strain Z-0088) and low electroconductivity of medium (from 44 μS to 0.8 mS).

The isolated organisms are subdivided into three groups according to their food requirements: saccharolytic bacteria (strains Z-0003, Z-0066, Z-0077, Z-0078, Z-0088), which use carbohydrates, acidotrophic bacteria (strains Z-0055, Z-0056), which use organic acids and methylotrophic bacteria (Hyphomicrobium sp.). Optimal concentration of substrat for representatives of acidotrophic group is 0.2 g/L. Concentration of a substrat above 10 g/l renders inhibitory action. The isolated bacteria are steady against antibiotics of a penicillinic group and group of cephalosporins which are produced mainly by fungi.

Thus the isolated microorganisms are representatives of group of acidophilic bacteria which use substances formed in the course of wood decomposition by xylolytic fungi as a source for carbon and energy. All investigated bacterial components of community are adapted for existence in ultrafresh conditions in close interrelation with fungi.

**Keywords** dystrophic waters; xylolytic fungi; dissipatrophic bacteria; oligotrophic bacteria.
Diversity and abundance of bacteria community associated with rhizosediment and un-colonized sediments in salt marshes of two Portuguese estuaries.

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The influence of salt marsh plants colonization (Halimione portucaloides) and the spatial and temporal distribution of prokaryotic was investigated in two Portuguese estuarine systems with different degrees of contamination: Cávado (41.5 N; 8.7 W) and Sado estuaries. In Sado, two salt marshes were preferred: Lisnave (38.4 N; 8.7W) and Comporta (38.4 N; 8.8W). In literature, Cávado River estuary was described as a contaminated estuary, Lisnave site was classified as a highly polluted site with a high impact potential and high risk to cause adverse effects on the biota and Comporta site was presented as a low contaminated site with low to moderated impact potential.

Total community DNA was extracted from 0.25 to 1 g of sediment or rhizosphere (colonized sediment) collected seasonally during one year. Diversity of bacterial community was evaluated by PCR-DGGE approach and quantitative real time PCR was conducted to determine bacterial 16S rRNA gene copy numbers. Total number of prokaryotic cells was also estimated by means of direct microscopic count of DAPI-stained cells. In addition, environmental characteristics (% of organic matter, grain size, and metals concentration - Pb, Ni, Zn, Cu, Cr, Cd, Fe and Mn) were related to bacteria assemblage composition.

Total microbial cells were found to be higher at Cávado estuary, with a general trend in all salt marshes of higher abundances in rhizosediment samples. Results revealed that high metal concentrations affect negatively the abundance of prokaryotic cells, since a negative correlation was observed between all metals tested and total cell counts. However, the increase of Zn and Cd levels were positively correlated with higher Shannon Weaver diversity index (H’).

Principal components analysis (PCA) based on the environmental variables, total cell counts and H’ identified two major groups. The first contained samples from Cávado estuary characterized by high DAPI counts and lower metals concentration (especially Zn, Cr and Fe). The second grouped all samples from Sado estuary with higher diversity index (H’). Hierarchical cluster analysis of all DGGE profiles did not show different diversity patterns between the three salt marshes. However, when DGGE profiles were analysed within each sampling site, samples were separated according to season, in Cávado estuary, and according to sediment type, in Sado estuary.

We can conclude that differences in salt marshes contaminant levels and the presence of salt marsh plants led to the selection of different microbial populations. Salt marshes may colonize large areas in temperate and subtropical estuaries and the presence of plants can modify the abundance and composition of the microbial community. Thus this influence should be taken into account in future studies to demonstrate the potential of the salt marsh plants for biological remediation and sustainable management of these ecosystems.

This work was partially funded by Fundação para a Ciência e Tecnologia (FCT), Portugal, through project POCTI/CTA/48386/2002.

Keywords: salt marshes; rhizosediment; microbial diversity; abundance

Diversity of bacteriophages and their hosts in the marine sediments: the combined approach using metagenomics and electron microscopy

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Bacteriophages (phages) are the most abundant life forms on Earth. Total number of viral particles is about 1031 and this is about one order magnitude larger than the bacterial communities. It is estimated that in the marine environment there is about 1010 viral particles per liter of sea water and 107 - 109 particles per kg of marine sediment. In our research, we combine a metagenomic approach that allows to investigate the sequence diversity of marine phages with electron microscopy techniques that allow for the assessment of morphological diversity.

The sea-floor sediment samples have been collected from three points of the Bay of Gdańsk (Baltic Sea) known to contain high concentration of EPS (extracellular polymeric substances), the main component of bacterial biofilm. The concentration of EPS were measured using fenol-acid-sulfate method. The method of isolation and purification of bacteriophages optimized in this work involved extraction with artificial sea water, centrifugation, filtration and precipitation in the presence of polyethylene glycol and sodium chloride and final extraction with chlorophorm. This method results in high concentration of viral particles which was confirmed by electron microscopy. We have observed several morphological types of bacteriophages; most of the isolated bacterial viruses have been found to belongs to Myoviridae, Siphoviridae and Podoviridae. The method for the construction of phage metagenomic libraries developed in this work allows to obtain about 1000-2000 plasmid clones from one sample with inserts 500-1500 nucleotides in length. Preliminary sequencing shows that high percentage of inserts is similar to known bacteriophage sequences. However, we have found that many sequences have been repeated in the library which suggests that parasquencing may be a much more cost-effective approach to obtain a large amount of metagenomic viral data. Our metagenomic approach is used in parallel with the efforts to isolate large number of bacteriophage-host pairs, which will be followed by sequencing of phage genomes. We hope that both approaches can complement one another and together allow for a more detailed characterization of the marine viral populations in the Baltic Sea.

Keywords: marine bacteriology, marine virology, bacteriophages, phage morphology, phage physiology, phage metagenomics.
Diversity of foaming producing nocardioform actinomycetes from wastewater treatment plants in Spain

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The problem of foaming in wastewater treatment plants (WWTPs) is a worldwide problem, and to become a better understanding what microorganisms causes this foam and to cure it, there is a need to identify the species present in the activated sludge basins. Microscopically, this foam usually contains large number of nocardioform actinomycetes containing mycolic acids, the so called mycolata. Mycolic acid-containing actinomycetes belong to suborder Corynebacterineae which contains the genera Corynebacterium, Dietzia, Gordonia, Millisia, Mycobacterium, Nocardia, Rhodococcus, Segniliparus, Skermania, Tsukamurella and Williamina grouped in seven families. In Spain the problem of foaming is not well understood and detailed investigations of nocardioforms in such foams has not been carried out. The aim of this study was isolate and identify mycolic acid-containing actinomycetes by analysis of 16S rRNA sequences to understand the biodiversity of these microorganisms in Spanish WWTPs with foaming problems.

Mycolata strains were isolated onto modified Czapek medium from twenty two WWTPs. DNA from pure culture colonies was extracted and 16S rDNA were amplified with universal primers. The 16S rDNA gene sequences were assembled using the PHYDIT program. The sequences were presumptively identified using the program BLAST. The almost complete sequences were aligned manually against sequences of reference strains of representative genus the suborder Corynebacterineae. Phylogenetic trees were inferred using the neighbour-joining algorithm from the PHYLIP suite programs and evolutionary distances matrices prepared. The topologies of the resultant unrooted trees were evaluated in a bootstrap analysis based on 1,000 resamplings of the neighbour-joining dataset using the PHYLIP package. The isolates of diatomomycins, mycolic acids and sugar composition of whole-cell walls were analyzed following standard procedures as well as phenotypic properties.

Twenty five representative isolated strains were selected in this work. All of them were filamentous, cocci or irregular rods Gram positive mycolic acid containing bacteria. All of the strains contained meso-diaminopimelic acid and arabinose, and galactose as characteristic whole cell sugars. Mycolic acids from the isolated strains co-migrated with those from the correspondent type genera of the genera Gordonia, Rhodococcus, Tsukamurella, Mycobacterium and Dietzia. Comparison of the nearly complete 16 rRNA nucleotide sequences from the isolated strains with corresponding nucleotides sequences of representative’s of the suborder Corynebacterineae confirmed that the isolates belonged to the species Dietzia maris, D. natri solutionis, Rhodococcus ruber, Gordonia spartu, G. polypseudovorans, G. terrae, G. malaguea, G. aurare, G. effusa, G. alkantivorans, G. jacobsae, Tsukamurella spumae, P. pseudopusa, T. tyrosinosolvens and Mycobacterium vanbaalenii. In all the cases, the nucleotide sequences similarity values among the isolated strains and type strains were between 98.52% and 100%. Results of the phenotypic test were coherent with the identification obtained with analysis of 16S rRNA sequences. From all the isolated studied in this work, the 48% belongs to the genus Gordonia, and the 30% belongs to the genus Tsukamurella. The rest of the isolates belong to Rhodococcus (4%), Dietzia (8%), and Mycobacterium (4%).

It’s interesting to emphasize that the nine Tsukamurella strains were isolated from nine different WWTPs showing the great importance that this genus may have in Spanish WWTPs.

To our knowledge, this is the first study about the diversity of mycolata in Spanish WWTPs. We have isolated fifteen different species of mycolata from twenty two activated sludge plants. These results showed a high diversity of mycolata in WWTPs. The results showed the isolation of mycolata species not related with activated sludge process such as Gordonia spartu, G. polypseudovorans or T. tyrosinosolvens. This is the first step to understand the complexity of the populations of mycolata in our country.

Keywords: mycolata; wastewater treatment plants; 16S rDNA

Acknowledgements This work was supported by grants from the Entidad de Saneamiento de Aguas de la Comunidad Valenciana, and Conselleria de Educacion y Ciencia de la Generalitat Valenciana.

Diversity of marine fungi on substrata collected in sandy beaches on Portuguese western coast

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Marine fungi are common in marine, estuarine and mangroves habitats worldwide. They are major decomposers of wood and herbaceous substrata, and their role is vital for marine ecosystems. In those environments a wide range of substrata are available for the colonization and growth of marine fungi.

Having Portugal a large coast with temperate waters it is important to survey the occurrence of marine fungi associated with the substrata found in sandy beaches.

In the present work, the diversity of marine fungi from 4 sandy beaches on west coast of Portugal was assessed during 8 months. At each field visit the abiotic parameters of sea water, temperature, pH and salinity were recorded. Out of 720 samples, intertidal wood and stems of herbaceous plant were collected for the detection of marine fungi, by direct observation of substrata. The identification of fungi was based on illustrated dichotomous keys of Kohlmeyer and Kohlmeyer (1979, 1991), Hyde and Sarma (2000) and Jones et al. (2009).

For the assembly of the surveyed beaches, 66 taxa were recorded (35 Ascomycota, 27 Anamorphic Fungi and 3 unidentified taxa) out of which 9 taxa were common to the four beaches surveyed: 6 Ascomycota (Corniopsis halina, Coriolospora maritima, Lignincola laevis, Luwworthia sp., Pleospora sp., Phaeosphaeria oreamaris) and 3 Anamorphic fungi (Halosporides varia, Phoma sp., and Stagonospora sp.). It was determined for each environment and type of substratum, the average number of fungi per substratum, frequency of occurrence of each taxa and diversity and similarity indices for marine mycota. One taxon (Coriolospora maritima) was frequent and all the others were infrequent. The average number of fungi per substratum and Shannon and Evenness indices were respectively 1.10, 3.47 and 0.83.

The pattern of geographic distribution of species was also registered and analyzed. Finally the collected data were compared with those from national and international works.

Keywords: diversity; marine fungi; sandy beaches; intertidal substrata

References
Effect of a respiratory inhibitor on the bioconversion of a xenobiotic by activated sludge

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The bioconversion of xenobiotics often involves oxidation-reduction reactions and one representative example is the reductive decolorization of azo dyes. The bioreduction of an azo dye (as model xenobiotic) by a mixed microbial culture (activated sludge) was thus chosen as a model system in the present study. The general aim was to investigate the effect of a respiratory inhibitor, namely sodium azide, on this bioconversion, when carried out by different microbial inocula.

In this study, the xenobiotic used was the azo dye Acid Red 14 and two mixed cultures were employed, one coming from an aerobic, laboratory-scale sequential batch reactor, fed with glucose, and another from the aeration tank of a municipal wastewater treatment plant. The bioconversion runs were carried out in closed recipients, with reduced headspace to induce anaerobic conditions, and using glucose as electron donor. All experiments were conducted in duplicate, and dye bioreduction was followed by UV-visible spectrometry. Dye adsorption onto the biomass was also measured, and accounted for 1 to 5% of the total quantified xenobiotic removal.

Dye bioconversion yield values above 90% were observed with both mixed cultures after 48 hours, in the presence or absence of added glucose, but bioconversion inhibition by azide showed different patterns, depending on biomass previous conditioning, i.e., freshly harvested or after aerobic incubation in the absence of carbon source (starved). Inhibition depended also on glucose availability during the bioconversion experiments.

Azide inhibition of dye bioconversion was observed with the fresh cultures from both sources, with 60 and 200mM inhibitor concentrations. When the biomass was previously starved (during 24 to 96h), both cultures showed a reduction in the azide inhibition effect. However, in the absence of glucose this alleviation of azide inhibition was not observed.

These results point to the existence of an alternative, dye bioconversion mechanism triggered in the starved biomass, in which the involved pathways are not inhibited by azide.

Acknowledgment: Financial support from FCT (Portugal), contract no. PDCT/AMB/59388/2004 is acknowledged.

Keywords: bioreduction; xenobiotic; azide; inhibition; activated sludge

Effect of Carbon and Nitrogen on the Predatory Behavior of Bacillus subtilis

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Bacillus subtilis demonstrates predatory behavior before cannibalism on other microorganisms that is not commonly observed in microorganisms under nutrient limitation to delay sporulation. However, cells of B. subtilis in Phosphate buffer solution (PBS) exhibits cannibalistic tendencies that is observed in microorganisms under stress. Under stress condition in PBS, B. subtilis behaves as a predator on other microorganisms. Since PBS includes no nutrients, the effect of carbon and nitrogen sources on the predatory behavior is obscured. A recent study by our group has suggested that B. subtilis utilizes one or more killing factor that lyse other microorganisms in predation and their sister cells in cannibalism when faced with nutrient limitation. In this paper, the effect of external carbon and nitrogen sources on the predatory behavior was studied. This study described that glucose as a carbon source was introduced into PBS in the absence of any other nutrients, the predatory behavior was delayed. This delay extended with the increase in the amount of glucose present in PBS. In addition the cannibalistic behavior after predation was also more delayed and extended in the presence of increasing glucose concentration. But in the presence of only ammonium sulfate in PBS as nitrogen source and was devoid of any carbon source, the effect of Predation was minimal. This work, thus, confirmed that Predation was more sensitive to carbon than nitrogen demonstrating that the predatory behavior may be more dependent on the energy sources in the medium than on nitrogen assimilation.

Keywords: Bacillus subtilis, predation, cannibalism, glucose, ammonium sulfate, MBRT.
Effect of spoil heap waters on *Anabaena* sp. – possible inhibition of cyanobacteria inoculum in new water body in the brown coal mining basin

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New water habitats were established in the post-mining area in the Sokolov brown coal basin, Czech Republic. The environment of new ponds and wetlands on spoil heaps and in former quarries is generally considered as potentially toxic for algae and cyanobacteria. Large range of pH (from 2.5 – 10), and high concentration of manganese up to 16.0 mg/L, iron up to 150 mg/L, and aluminium up to 60 mg/L are typical for these types of water.

In the former largest quarry Medard, there have been established a new waterbody with planned depth of 51 m, area of 501 ha. It has been filled with water from the river Ohře since 17th July 2006. Water chemistry of the new water body might be influenced by discharged water from the surrounding heaps. Possible inhibition of cyanobacterial inoculum by heaps waters is the main task of this paper.

We studied the possible toxicity effect on PSII of Cyanobacteria *Anabaena* sp. of waters from seven small localities with the pH range 2.6 – 8.4, conductivity 2 900 – 10 800 μS/cm. We analyzed the water samples from the 0, 5, 10, 15 m depth of Medard water body as well. After one hour of illumination of 300 μmol/m²/s, we used the fluorescence light curve, electron transport rate and efficiency of photochemistry (Fv/Fm) measurement to examine the toxicity effect on photosystem II (PSII) of culture of *Anabaena* sp.

When the cyanobacterial culture was mixed in ratio 1:19 (dilution to 5%) with water from four most acidic localities (pH 2.6 – 3.0), *Anabaena* sp. showed no photosynthetic activity. After one hour of illumination, the Fv/Fm value fell to 0 % (60 %) of the control Fv/Fm value in the 2.5 % (1 %) dilution. The efficiency of photochemistry was higher in the medium of the pH 4.40 (20 %) and in the 75 % dilution of the surface water of Medard water body (88 %). The water from localities with pH 7-8, and conductivity 4 700 (9 800) μS/cm seemed to have no change on fluorescence of PSII.

The water from Medard with the pH between 5.92 – 6.93 and conductivity 264 – 546 mS/cm was far less toxic effect than from acidic spoil heap waters – not until 75% dilution showed any effect on its photosynthesis. The highest sink of Fv/Fm value was observed in the depth of 10 m (25 % compared to control), in contrast to the samples from 15 m (96 %), 0 m (88 %) and 5 m (100 %).

The most negative effect on the PSII is the environment with both low pH and high amount of dissolved ions. The dissolved ions themselves seem to have less effect on PSII. The diminution of 300μl multiplied the decrease of the photosynthetic ability.

Keywords: acidic waters, post-mining area, *Anabaena* sp., Medard water body

Effect of the ammonium chloride concentration on the mineral medium composition – Biodegradation of phenol by mixed culture

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Phenol and its homologues are aromatics containing hydroxyl, methyl, amide and sulphonic groups attached to the benzene ring. These molecules are both anthropogenic and xenobiotics [1]. Phenols are environmental pollutants discharged through wastewaters from fossil fuel refining processes, phenol manufacturing plants, pharmaceutical and a variety of other industries [2]. Phenols are toxic to several biochemical reactions. However biological transformation of phenols to non-toxic entities exists in specialized microbes, owing to enzymatic potential involving enzymes of aromatic catabolite pathways.

A series of experiments were performed to examine the effects of the mineral medium composition and the pH on phenol removal. In this purpose, phenol biodegradation was carried out in a batch reactor containing mixed bacteria; the temperature (30°C), the stirring velocity (200 r/min) and the phenol concentration (100 mg/L) were kept constants. The initial pH was varied in the range 5 – 8 and the mineral components were tested in the following concentration ranges: 0.25 – 2 g/L for NH4Cl, 1 – 4 g/L for KH2PO4, 1 – 4 g/L for NaH2PO4 and 0.05 – 0.2 g/L for MgSO4. Their effects on phenol biodegradation and specific growth rate were examined. All experiments were carried out at a given initial bacterial concentration of 0.08 g/L (based on optical density determination, 0.079).

Maximum specific growth rate (0.65 h⁻¹) and total phenol removal (99.99 %) were recorded for an optimal pH value of 8 and the following mineral medium concentrations (g/L): 1, 3, and 0.1 for NH4Cl, KH2PO4, NaH2PO4 and MgSO4, respectively.

Bacterial growth kinetics were described according to the Riccati model, the correlation coefficients were in the range 0.89 to 0.97. The constant rate k and the constant related to the percentage of inhibition β were determined according to the initial pH of the solution and the mineral medium concentrations.

Keywords: Biodegradation; Phenol; Mixed bacteria; Kinetic; Riccati model.

References
Effect of Toluene-containing Synthetic Wastewater on Archaeal Population Dynamics in Anaerobic Sequencing Batch Reactors

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Toluene, which is an aromatic hydrocarbon is widely used as a solvent in several industries. Solvent-containing wastewaters are generally treated using anaerobic bioreactors due to high organic content. However, there are still some concerns in the application of anaerobic treatment processes for this kind of wastewaters due to possible detrimental effects of the compounds on microbial community playing crucial role in anaerobic digestion. Improvement of anaerobic systems for treatment of the wastewaters needs determination of inhibitory effects of the compounds on microorganisms’ vitality and activity. Therefore, in this study, a lab scale anaerobic sequencing batch reactor (ASBR) fed with toluene-containing synthetic wastewater has been monitored in terms of changes in methanogenic Archaea. In addition, a control reactor was operated with synthetic wastewater without toluene. The reactors were inoculated with a sludge having high methanogenic activity of 453 mLCH4/gVSS/day. The ASBR was operated with one cycle/day including the following four discrete steps: a feed step (0.5 h), a react step (22 h), a settling step (1 h), and a decant step (0.5 h). Under these operating conditions, the S/N ratio was 0.59 gCOD/gVSS. VSS concentration in the reactor ranged between 6000-6530 mg/L during the operation period. The microbial community structure was characterized using Fluorescent in situ Hybridization (FISH) with ribosomal RNA targeted oligonucleotide probes specific for Bacteria, Archaea and phylogenetically defined groups of methanogens and quantified using SPOT RT software. The reactors were fed with glucose based synthetic wastewater and then toluene was added to the synthetic wastewater. At the beginning of the operation period with toluene, COD removal efficiency of the reactor was not affected by toluene addition. However, after repeated introduction of toluene at a concentration of 0.3 mM to the synthetic wastewater resulted in a decrease in reactor performance in terms of COD removal efficiency and the size of archaeal community. After day 34, a gradual deterioration in the reactor performance was observed with a COD removal efficiency falling to 70%. A sudden decline in the performance of the reactor was observed after day 52 with a COD removal efficiency of 40%. At the same period, methane yield was approximately 0.17 m3CH4/kgCODinout. The most pronounced effect among methanogenic Archaea has been observed in Methanomicrobiales species. Although this group was approximately 7% in total active population in the seed sludge, after feeding the ASBR with toluene-containing synthetic wastewater, this group has not been observed in the sludge samples taken from the reactor. At the end of the operation period of the reactor, dominant species in total active population were Methanoseta (12.3%), Methanobacteriales (5.6%), Methanosaeta (3.6%) and Methanococcales (1.4%) respectively. Numerical dominance of the genus Methanoseta compared to other methanogens in anaerobic reactor exposed to toluene has been observed. In the control reactor, dominant species (in total active population) were Methanoseta (15.5%), Methanomicrobiales (10.0%), Methanobacteriales (9.6%), Methanosaeta (7.8%) and Methanococcales (5.5%), respectively.

Keywords: Anaerobic, ASBR, bioreactor; FISH, toluene, methanogenic Archaea

Effect of wastewater treatment plant effluent on the coastal waters of Peniche – A preliminary microbiological study

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Peniche is an important tourist destination from the western region of Portugal and was not found information on the wastewater plant effluent impact on the sea water. Marine pollution levels were analyzed in three sample locations: (i) at the exit of the wastewater treatment plant (WWTP), (ii) at the closest beach north of the WWTP (Papoa), and (iii) at the closest beach south of the WWTP (Carreiro de Joanes). Sampling occurred during April and May 2009. The marine pollution levels were achieved by the enumeration of total heterotrophic aerobes (THA), through the presence of coagulase positive Staphylococcus was determined by a conventional plating method recommended by Biorad for environmental samples analysis and the occurrence of Salmonella was analyzed through a comparison between a conventional plating method and the Enzyme Linked Fluorescent Assay (ELFA) performed in Mini-Vidas. The maximum values for THA were obtained on the effluent exiting WWTP (> 5,5 x 108 ufc/mL). Likewise, in this site, E. coli was presented higher levels which exceeded the maximum recommended by the Directive 2006/7/CE and by the Portuguese Legislative Act n°236/98 (300 100/mL). However, the other areas under study (Papoa and Carreiro de Joanes) presented lower values, Enterococcus was only detected in the WWTP site, where it presented values (≥ 1100/mL) above the recommended ones (185ufc/100mL) according to the Directive 2006/7/CE. The presence of Staphylococcus was observed in all the tests carried out at WWTP. In the remaining studied areas, it was only detected in the Carreiro de Joanes. As for the Salmonella analysis, the detection was only confirmed outside WWTP. Clearly, the effluent exiting the WWTP of Peniche presents unsatisfactory microbiological values for water quality. Nevertheless, these pollution levels not seem to affect the closest beaches to the WWTP. The comparison between the ELFA and conventional methodologies was inconclusive, more analyses are necessary in order to confirm the ELFA method suitability for testing complex environmental liquid samples as the coastal waters.

Keywords: Coastal water quality, Waste Water Treatment Plant, Salmonella


doi:10.1002/bimr.201000002

45 46
Electrochemical process coupled with biological treatment for the removal of a pesticide, phosmet.

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In intensive agricultural practice, repeated use of pesticides may result in more frequent occurrence of agrochemicals in raw water resources. Some effluents of agricultural activities (unused treatment solutions, spray, machine and pesticide container washing) contribute to water resource pollution. Pollution of water with biorecalcitrant organic compounds is becoming increasingly worrying and pesticides remediation from environment is now a great challenge for the scientific community.

The aim of this study is to assess and develop an integrated process in order to degrade phosmet, an organophosphorous pesticide widely used in insect pest management for crop production.

A study was carried out on the biodegradability of phosmet with activated sludge from a local municipal wastewater treatment plant and by means of BOD5 measure. The results showed that this organic compound was not biodegradable in our operational conditions. Moreover, Microtox test showed that the target compound presented a high toxicity for Vibrio fischeri bacteria. So, a conventional biological treatment is not appropriate for phosmet polluted effluents.

One way to remove phosmet and in the same time minimize the operational cost is to develop an efficient pre-treatment process that reduces the toxicity and/or increases the biodegradability of the effluent containing the target compound before a low cost biological treatment.

Electrochemical behavior of phosmet was studied by cyclic voltammetry with a vitreous carbon electrode. Phosmet was reduced in neutral medium; the feasibility of an electrochemical pre-treatment was thus demonstrated. The setup used to perform electrolyses includes graphite felt with a high specific area as working electrode in a flow cell. After potentiostatic electrolysis (-1.3V/ECS), phosmet was not detected by cyclic voltammetry. H-NMR spectra and thin layer chromatography pointed out many by products. H-NMR spectra of the main by-product showed the absence of the aromatic ring; only the phosphorus part of phosmet has been identified. Furthermore, phosmet-oxon, a very toxic derivative, was not observed. The electrolyzed solution was characterized by a lower toxicity and a higher biodegradability compared to the target molecule. Indeed, the EC50 value increased from 7 % initially to 58 % after electrolysis and the BOD5 value increased from 4 mg L-1 initially to 9 mg L-1 after electrolysis, leading to an increase of the BOD5 on COD ratio from 0.19 to 0.42, namely above the limit of biodegradability (0.4). These encouraging results were confirmed during activated sludge culture since an almost total mineralization of the electrolyzed solution was recorded (97 %).

Key-words: Integrated process, electrochemical pre-treatment, biological treatment, pesticide degradation, phosmet

Enrichment of Polyphosphate Accumulating Organisms in Accumulibacter Clade I: affinity for nitrate, nitrite and oxygen

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Enhanced Biological Phosphorus Removal (EBPR) has been suggested as an effective alternative to chemical precipitation methods for phosphorus removal. The coupling of this process with denitrification, by using a population capable of removing phosphorus using nitrate/nitrite as electron acceptor, reduces the amount of carbon needed and also the sludge produced as compared with two distinct processes.

This coupling has been suggested since Candidatus Accumulibacter phosphatis, one of the main Polyphosphate Accumulating Organisms (PAO) was shown to be able to denitrify. However, it remained unclear whether all PAOs could denitrify or if there were Denitrifying PAOs (DPAOs) and non-DPAOs. Furthermore, metagenomic studies performed on Accumulibacter-PAO enriched cultures revealed that their genome did not encode for the nitrate reductase, suggesting the incapacity to convert nitrate to nitrite and only being able to use from nitrite onward (Martin et al., 2006). However, Carvalho et al. (2007) suggested that there was a greater diversity of metabolic capacities within Accumulibacter than expected initially by obtaining two different Accumulibacter morphologies with different affinities for nitrate. Flowers and He et al. (2008) then designed two Fluorescence in situ Hybridization (FISH) probes that were able to distinguish between clade IA (Acc-I-444) and clade IIb, IIC and IID (Acc-I-444) within Accumulibacter.

A reactor was set up using inoculum sludge from a parent EBPR lab-scale reactor working in anaerobic/aerobic conditions with propionate as the carbon source. The reactor was progressively adapted to anaerobic-anoxic-aerobic conditions during 150 days. This acclimatization process consisted in a gradual increase of the anoxic period from 0 to 4 h and a decrease in the aerobic period from 4 to 1 h, as well as an increase of the nitrate concentration given in one pulse at the beginning of the anoxic phase from 0 to 50 mg N/L. This acclimatization was monitored not only through chemical analyses (PO4-, NO2-, NO3-, glycogen, propionate and polyhydroxyalkanoates (PHA)) but also through microbial characterization using the Fluorescence in situ Hybridization (FISH) technique.

Throughout the 2 years of operation the capacity of the population to use nitrate or nitrite (50 ppm-N) and oxygen as electron acceptors was tested in batch experiments. At the highest enrichment state (>95% Accumulibacter clade I) this population was able to use nitrate, nitrite and oxygen as electron acceptor, which supports the hypothesis that this Accumulibacter clade is a nitrate-DPAO. Although nitrate and nitrite are both used, oxygen leads not only to faster phosphorus uptake (4.5 times higher) but also to higher PHA degradation and higher glycerol storage. In tests using nitrate, nitrite accumulation was never observed suggesting that nitrate conversion to nitrite is slower than from nitrite to nitrogen gas. Though nitrite led to a higher phosphorus uptake rate than nitrate the P/N ratio for nitrite was 2 times higher than the one with nitrite suggesting that nitrate is better electron acceptor than nitrite.

These results confirm that a population enriched in Accumulibacter clade I presents a versatility towards different electron acceptors, being able to take up phosphorus using oxygen, nitrate and nitrite.

Key-words: EBPR, DPAO, Candidatus Accumulibacter phosphatis, denitrification

Environmental impact on *Rhizobium* sp. cells

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The soil bacteria *Rhizobia* are the most promising and agriculturally important group for the accomplishment of biological nitrogen fixation leading to the formation of symbiotic nitrogen fixing nodules.\(^1\) They can convert atmospheric nitrogen into ammonium compounds which can be readily absorbed by the plants system. *Rhizobia* provide the major biologic source of fixed nitrogen in agricultural soils.

Crop producers often add chemical fertilizers in the soil to enhance growth and vigour of crop of choice. Indiscriminate use of fertilizers and non point source run off lead to accumulation of nitrate, phosphates and potassium in the soil which may be harmful to the soil inhabitants. Pesticides are predominantly used in agriculture for control of insects, weeds and other pests. As pesticides are not species specific their biological activity gets extended to wide variety of non target organisms including man itself. Endosulfan, an organo-chlorine class of pesticides, is used widely in the Indian sub continent which is acutely toxic to the various organisms.\(^2\) Dithane M-45 a fungicide is used to control pests in mung bean plants. Since *Rhizobium* sp. has immense importance in agriculture, any adverse effects induced by environmental factors seem to be a matter of interest.

Microbiological studies revealed that various nitrogenous and phosphate based fertilizers and commonly used pesticides have detrimental effects on the growth and culture of *Rhizobium* sp. cells. Electron microscopic studies depicted significant structural alteration at cellular level. [Fig a,b]

Our investigations clearly indicate that both fertilizers and pesticides at the doses used in agricultural practices have considerable adverse effects on *Rhizobium* sp. cells. Endosulfan is reported to induce different cellular toxicity to different organisms including man. For the first time we have reported that in bacteria also, endosulfan has predominant effect.

![Fig a: Control, b: Endosulfan treated.](image)

References

Estimation of freshwater cyanobacteria primary production with DCMU-fluorescence method

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Estimation of cyanobacterial primary production (PP) is important for studying energy flow in aquatic ecosystems. Moreover, planktonic cyanobacteria can cause toxic blooms harmful for human and animal health. There have been relatively few studies of cyanobacterial PP. We suggested to use an original DCMU (3,3',4-dichlorophenyl)-1,1-dimethylurea) fluorescence method for estimation of planktonic cyanobacteria primary production. A special fluorometer equipped with a system of replaceable filters is used to differentiate three regions of the spectrum (410±20, 510±20 and 540±10 nm) that can excite the basic light-harvesting pigments. For the estimation of PP (gO2/(m3·h)) a formula was proposed: PP=0.006·(ΔF/Fm) ·Chl a·√I, where ΔF/Fm – potential photosynthetic activity, Chl a – concentration of chlorophyll a (mg/m3), I – average intensity of photosynthetically active radiation (W/m²).

During the experiment in Siberian reservoirs the dominant species were Microcystis sp., Anabaena flos-aquae (Lyng.) Breb., Aphanizomenon flos-aquae (L.) Ralfs. Positive significant correlation between the results of DCMU-fluorescence method and in situ bottle experiment was found. The obtained PP values for had a high significant correlation with the results of in situ experiments for such studies.

Keywords Periphytic cyanobacteria, chlorophyll fluorescence, primary production

Extraction of nucleic acids from trichloroethene (TCE) degrading bacteria in extremely clayey groundwater sediments

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Extraction of nucleic acids from clayey environmental samples has proven to be extremely problematic. Phosphor bindings between the nucleic acid and clay minerals such as kaolinite cause the nucleic acid to be bound tight to the sediment particles and thereby prevent extraction. For the present project our aim was to extract DNA and mRNA from a microbial community that is degrading TCE in clay rich groundwater sediment. Especially we were interested in extraction of mRNA of the functional gene vcrA. The vcrA gene encodes a vinylchloride reductase that performs the final step in the degradation pathway of TCE. Therefore, the presence of vcrA mRNA can be used as an ideal biomarker for ongoing complete dechlorination processes being carried out.

In order to investigate the specific sorption effects between nucleic acids and our sediment, we performed a standard sorption experiment with 3H-labelled DNA. Compared to other sediments with less clay and more organic matter, we observed a very pronounced sorption in our clayey sediment. Therefore we tested numerous reagents in order to reduce the effects of sorption, and found the G1 reagent to reduce sorption most.

To test the G1 reagents influence on DNA and mRNA extraction we set up a microcosm experiment with 100 g sediment, 200 ml water and 10⁷ cells of the commercial dechlorinating culture KB-1 ml-1. The addition of vinylchloride ensured a proper expression of the vcrA gene and samples were taken from the microcosms at appropriate timepoints. The addition of G1 reagent to the sediment prior to DNA and mRNA extraction gave significantly larger yields. The Mobio UltraClean soil kit was used for DNA and a modified protocol of the mRNA extraction method described by Nicolaisen et al. (2008) was used for mRNA extraction.

Keywords DNA/mRNA extraction, DNA/mRNA sorption to clay, TCE dechlorination, KB-1 culture, vcrA genes

References
Factors affecting the Internal Gelation System as immobilization method for the isolation and cultivation of anaerobic microorganisms

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It has been estimated that 99.9% of all microorganisms are still unculturable. Therefore, the development of new tools for addressing this drawback is necessary. Anaerobic microorganisms show high potential for biotechnological applications, however, they constitute one of the most uncultured groups among the microbial diversity.

The aim of the present work was to determine the most significant factors that can influence the Internal Gelation System (IGS) as immobilization method for anaerobic microorganisms isolation and cultivation.

Microbial consortia able to degrade cellulose material were cultured according to Hungate anaerobic method. Well-grown microorganisms were encapsulated under anaerobic conditions in alginate beads in accordance with the IGS using two different oils, beakers with and without baffles and rotation impeller speed at 100 and 1000 r.p.m for beads formation. The formed alginate beads were measured as well as determining the shape (regular or irregular) and analysing the results by means of ANOVA.

ANOVA analysis showed that oil types used combined with the rotation impeller speed are the most significant factors for formation of regular shape beads. The presence of baffles do not affect on the shape. To obtain beads in a range between 20-80 μm, the conditions for immobilization also depend on the oil type in combination with the rotation impeller speed. The presence of baffles showed significance on the size of beads when analysed alone.

The best conditions for immobilization of anaerobic microorganisms depend on the oil type used and the rotation impeller. The presence of baffles can favour the formation of smaller beads, when it is required. One to three bacterial cells was microscopically determined inside the beads. These beads might be grown in specific media to increase biomass. Using this IGS, it would be possible to isolate new organisms, earlier not possible to cultivate in the lab with traditional techniques.

Keywords: immobilization, alginate beads, Internal Gelation System, anaerobic culture

First data on the growth of marine biofilms dominated by Ostreopsis spp. (Dinophyceae) in microcosm experiments

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The requirement of intervention for Harmful Benthic Algal Blooms (HBABs, Congestri et al. 2006) by public officers and scientists has increased alarming in Italian coastal areas since 1998 when blooms of the toxic dinoflagellate Ostreopsis spp. were first recorded in Tuscany (Middle Tyrrhenian Sea). More recently HBAB impacts on the marine ecosystem such as hypoxia, anoxia and invertebrate mortalities and human health with hundreds of hospitalisations from skin irritations, fever, respiratory affections and conjunctivitis have been serious in Italy. These incidents have been explained by the presence of a palytoxin analog, ovatoxin, produced by O. ovata in both water and aerosols. After Italian reports of Ostreopsis blooms there was increased interest in the whole Mediterranean basin and some reports exist from Brasil and New Zealand this year. Blooms of Ostreopsis spp. do not seem to follow known trends of other planktonic blooms, and thus leave many open questions as to their outbreaks, and their dynamics, and complicate management strategies in the short- and long-term.

In the framework of a national research program (Ostreopsis ovata and Ostreopsis spp.: new risks of microalgal toxicity along the Italian coastline – Italian Ministry of Environment) to improve the knowledge of Ostreopsis HBAbs, a laboratory microcosm approach was used to grow microphytobenthic communities, sampled during bloom incidents, under controlled conditions. HBAbs occur in nature as thick mucilaginous biofilms covering both biotic and abiotic substrates during the summer months along the middle Tyrrhenian Sea coast. Within the biofilm Ostreopsis spp. are associated with other toxic dinoflagellates Amphidinium ccarteae, Coolia monotis and Prorocentrum lima, along with a variety of benthic diatoms, mostly pennates, and filamentous cyanobacteria. All the cells of the biofilm are embedded in a common mucilage matrix (EPS, exopolymers substances). Dispersal of cells occurs during blooms when mucilage flocs and cells can detach from substrates and are carried with currents.

The inocula for the incubator experiments were taken from two sites in the Lazio region in July and August 2009 (Porto Romano, Formia and Sperlonga, Latina). At these sites the biofilms started to form on bryozoans and macroalgae, attached to hard substrata, at around 20-30 cm depth. Bryozoans and macroalgae were collected, and the cells were washed off and collected, this was then filtered in order to prepare cell suspensions to inoculate the flow lane incubator. Cell aggregations on the polycarbonate slides formed within 10 days under the culture conditions tested (25°C. 110 µmol photons m⁻² s⁻¹ and 50 L h⁻¹ flow velocity of K/2 medium). The distribution of cells was patchy with filamentous forms of cyanobacteria and colonial diatoms in mucilage tubes visibly streaming in the flow direction. Growth curves indicated that biofilms reached a mature stage in 40 to 50 days. Qualitative and quantitative analyses were performed on biofilm samples collected at three stages of development (these stages were defined by percent light absorption by the biofilm: initial, 10%; active, 50%; mature, 90%). In one experiment detachment of biofilm from slides was observed around day 37 of the incubation period EPS analyses may enable the interpretation of this event as a function mucilage composition change. Confocal microscopy of biofilm integrity is used to identify the initial processes of cell adhesion to substrata. Preliminary light and epifluorescence microscopy showed shifts in biofilm composition and prevalence of cyanobacteria in the late growth stages, although concentrations of chlorophyll a, b and c indicated a stable contribution of diatoms to the phototrophic biomass over the experiments. Cell countings will further contribute to understand biofilm development and species succession under simplified, stable environmental conditions.

Keywords: Ostreopsis spp., biofilms, microcosm

Formation of macro- and micro-sized copper sulfide particles by pure cultures of metal-resistant sulfate-reducing bacteria

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Metal precipitation as sulfides by sulfate-reducing prokaryotes (SRP) is an important process in various technological schemes to treat metal-polluted wastes. Some aspects of chemical speciation and mineralogical composition of biogenic sulfides are still poorly understood. Studies of copper sulfide production by pure SRP cultures have been hampered by copper toxicity to bacteria. We used copper-tolerant SRP pure cultures isolated earlier from different metal-polluted environments to characterize biogenic sulfides produced in short- and long-term experiments in a chemically defined growth medium. Most SRP described to present are either Gram-negative Desulphatebacteria or Gram-positive Firmicutes. Copper-tolerant strains belonging to both phylogenetic groups including Desulfosporosinus sp. DB, Desulfomicrobium sp. KN, Desulfovibrio sp. R2, and Desulfotalea sp. A2 were used in experiments.

Scanning electron microscopy with energy-dispersive x-ray analysis (SEM-EDAX) revealed that copper, sulfur, and iron were the main constituents of the particles produced by bacteria. The molar ratio of the elements changed depending on bacterial strain and incubation time. Carbon, oxygen, and phosphorus also appeared on the EDAX spectra. Powder X-ray diffraction (XRD) analysis indicated that covellite (CuS) and chalcocite (CuFeS2) were the major crystalline phases in the precipitates obtained from bacterial cultures. Chalcocite dominated in the precipitates retrieved from long-term experiments. Whereas covellite was the major phase observed under the short-term incubations. The XRD patterns showed presence of minor amounts of various copper and iron sulfides including, pustatinite (CuFeS4), yarrowite (Cu9S8), geerite (Cu8S5), bornite (Cu5FeS4), marcasite (FeS2), and kansite (Fe9S8). Biogenic precipitates also contained iron and copper phosphates and hydroxyphosphates.

Transmission electron microscopy (TEM) analysis showed that studied Desulfotalea and Desulfosporosinus cells were coated with finely divided small particles or clusters of particles. The particle size was approximately 10-20 nm. Nanosized crystalline particles, approximately 30 nm in size, were detected in cell-free supernatant after the cells were destroyed by several freeze-thaw cycles. The electron diffraction pattern of the particles was consistent with chalcopyrite (CuFeS2) lattice. Thus the cell wall may be considered as an initial nucleation site for the nanosized copper sulfides which undergo subsequent aggregation with time.

We acknowledge support from the Russian Fund for Fundamental Research (Grants 09-04-99138-r_ofi and 09-04-90734-mob_sfi) and Fund for the Development Assistance, Program “UMNIK” N 6362p/8761 to OB.

Keywords chalcocite; covellite; Desulfotalea; Desulfomicrobium; Desulfosporosinus; nanosized metal sulfides; sulfate-reducing prokaryotes; SEM-EDAX; TEM; XRD

Freeze-thaw pre-lysis may not improve microbial DNA extraction from sponges

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Sponges (Porifera) are of the oldest metazoa. Virtually all sponges are sessile filter feeders. Many sponge species have developed complex defence mechanisms to protect them from predators and to compete with other species for bottom substrate. Many of the substances employed by sponges for defence or competition are produced by microbial endosymbionts. For example, several sponge microbial symbionts are able to produce effective antiviral, antimicrobial and cystostatic components of fundamental importance for the maintenance of sponge health. Microbial symbionts are also involved in crucial physiological processes such as metabolic waste processing. Recently several studies have applied molecular tools (culture-independent techniques) for a more in depth characterization of sponge microbial diversity. However, these techniques are highly dependent on the efficiency of the microbial DNA extraction method. Total microbial community DNA associated with sponges has been traditionally extracted using liquid nitrogen in order to pulverize the strong calcareous/siliceous sponge skeletal structure. However, whether or not the liquid nitrogen treatment affects the sponge associated microbes has not yet been addressed.

The aim of this study was to compare the microbial community diversity associated with the sponge species Cinachyrella sp. and Placospongia sp. after genomic DNA extraction with and without liquid nitrogen. The liquid nitrogen pulverization step was followed by microbial DNA extraction with the UltraClean Soil DNA Isolation Kit (MO BIO Laboratories, Inc), using a protocol modified to include a physical disruption step with the FastPrep bead beating system. After PCR amplification of the V6-V8 region of the 16S rRNA gene, bacterial structural diversity was assessed by Denaturing Gradient Gel Electrophoresis (DGGE).

Analysis of DGGE band profiles revealed differences in the bacterial community structure detected with and without the use of liquid nitrogen. The liquid nitrogen pulverization step was followed by microbial DNA extraction with the Ultrapure Soil DNA Isolation Kit (MO BIO Laboratories, Inc), using a protocol modified to include a physical disruption step with the FastPrep bead beating system. After PCR amplification of the V6-V8 region of the 16S rRNA gene, bacterial structural diversity was assessed by Denaturing Gradient Gel Electrophoresis (DGGE).

The XRD patterns showed presence of minor amounts of various copper and iron sulfides including, pustatinite (CuFeS4), yarrowite (Cu9S8), geerite (Cu8S5), bornite (Cu5FeS4), marcasite (FeS2), and kansite (Fe9S8). Analysis of DGGE band profiles revealed differences in the bacterial community structure detected with and without the use of liquid nitrogen. Furthermore, we observed a reduced diversity of the bacterial community in the samples processed with liquid nitrogen, indicating that this extraction method might underestimate the diversity of sponge-associated microorganisms.
From pure cultures to bacterial communities: Interaction of Microarrays and Next Generation Sequencing (NGS)

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Metatranscriptomics offer the opportunity to explore the gene expression of bacterial communities in their natural environment. So far the majority of marine microorganisms are only poorly understood, although they represent more than 90% of the world’s prokaryotes. They play a vital role in the global carbon and nitrogen cycles and are expected to be a treasure trove for new genes interesting for biotechnology and medical applications. For Metatranscriptomics Next Generation Sequencing (NGS) techniques are the tool of choice. Especially long read machines like the Roche 454 Titanium pyrosequencer allow digging into the expressed genetic repertoire of a so far unknown sequence space. This is a clear advantage over classical microarrays approaches.

But the information gained will only be informative if the function of a sufficient number of the detected genes is known. In order to enrich this knowledge, gene expression profiling of pure cultures growing under defined conditions is essential.

In this contribution, the results of two approaches, both parts of the Microbial Interactions in Marine Systems (MIMAS) project, will be presented:

1. gene expression profiling of the marine planctomycete Rhodopirellula baltica SH1 with a whole genome microarray.2 Already in 2003 the complete genome sequencing of R. baltica SH1 revealed many fascinating and rare features like a high number of sulphatases, genes for a C1 metabolism and a global mechanism of gene regulation1.

2. the analysis of the Metatranscriptome of marine prokaryotic communities in the North Sea using long read Next Generation Sequencing approaches.

The overall aim of the MIMAS project is to explore the seasonal changes of the microbial communities at long term ecological research sites in the North Sea (Heligoland Roads) and the Baltic Sea (Gotland Deep). An integrated, state of the art, approach will be applied using the full spectrum of currently available molecular tools: rRNA sequencing and single cell in situ hybridization to unravel the diversity and abundance of organisms, Meta genomics to address the genetic potential, as well as Metatranscriptomics and Metaproteomics to explore the active set of genes with respect to seasonal changes.

References


Functional profile of fungi affecting cultural heritage in archives and libraries

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Filamentous fungi colonize different organic and inorganic materials and play an important role in biodeterioration processes. They can tolerate desiccation, high salt concentrations and heavy metal compounds that are present in inks and pigments and are thus frequent inhabitants on paper supported objects.

The fungal and bacterial communities that can develop on a book are similar to the communities of decomposers that, in natural environments, transform nutrients bound in dead organic matter into low molecular or inorganic forms, making them available to plants. The development and maintenance of a fungal community on a shelf of a library or in a single book depends on the spores that reaches the material’s surface, on the microenvironment (temperature, relative humidity, light), on the water activity of the substrate, on the casual events that helps colonization of materials (insect dispersion, human contamination, external sources of fungal diversity). A library or a single book can be compared to a virgin land that can be reached by some colonizing organisms that behave like pioneer species on a nude soil. For Wardle (2002) and Mikola et al. (2002) species identity and composition of decomposers have a far larger impact on ecosystem processes than species richness per se. When considering paper stored in a closed environment, its colonization and biodeterioration depends from species identity and composition since only cellulolytic organisms can exploit the bulk of the substrate.

Like in natural environments the diversity-functioning relationship is driven by the presence or absence of key species that are able to differentiate and species interaction. Resource partitioning or facilitative (or negative) interactions between species affect the substrate exploitation process in natural environments as well as in artificial ones. The study of the mechanisms underlying the microbiological attack of historical materials has been widely practiced and still represents one of the main focuses of those institutions and laboratories that are involved in cultural heritage conservation. Many studies on the role of micro-organisms in the defacement of cultural heritage utilise standardised laboratory models to establish, under controlled conditions, which species of fungi or bacteria colonise a given substrate, and therefore do not raise the problem of working with objects of art that cannot be cut, sampled, or subjected to invasive analysis.

Although knowledge of the functional diversity and the metabolic characteristics of moulds is important in both the prevention and treatment of biodeterioration of cultural heritage, very few in-depth studies have been carried out on the subject until now.

In this study Biolog FF microplates were used to obtain the metabolic fingerprint of filamentous fungi responsible for biodeterioration in library materials. As part of a survey made in an historical library in Rome, a significant fraction of moulds were collected from volumes showing evidence of attack by fungi. Fungal strains were also isolated from the air using an impaction sampler (SAS), and from the walls, where colonies were clearly visible.

The fungal strains were purified of bacterial contaminants, separated and identified. Biolog FF microplates were then inoculated with each strain according to Biolog’s Manual protocol. The plates were read using a Biolog Microplate spectrophotometer at 490 and 750 nm. Raw data (optical densities) were transferred to an Excel (Microsoft®) sheet according to sample (fungal strain), replicate (3 replicates each), and reading time (10 reading points, one every 24 hours), although for each strain only the values at the relevant plateau of the colour development curve were chosen for statistical analysis. Following a background correction, average values for six categories of substrates were calculated (polymers, carbohydrates, carboxylic acids, amines and amides, amino acids, miscellaneous). Discriminant Analysis was employed to investigate the differences in the metabolic fingerprint among fungi according to two classifying categories: the sampled material (leather, fabric, air, plastic), and the sampled area of the library (upstairs, first floor, ancient books section). When using polymers, carbohydrates, and carboxylic acids as variables in the analysis the fungal strains, a significant classification clustering was obtained. A statistically significant separation of fungal strains was also obtained for the sampled areas, indicating that different zones of the conservation environment under examination were colonised by fungal communities with a different functional profile. Further studies are being focused on the analysis of fungal metabolic characteristics which directly relate to important processes that take place in cultural heritage biodeterioration, and to the identification of a set of substrates that can better indicate the potential of fungal strains to decompose specific materials.

Keywords: fungal communities, metabolic profiling, cultural heritage, indoor environment
Fungi – bacteria interactions in the oxalate carbonate pathway: an approach using microcosms

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Calcium salt of oxalic acid is an important metabolic product of plant and fungi in natural environments. Its decay allows calcium carbonate to precipitate in normally carbonate-free tropical acidic soils. This results from calcium oxalate oxidation by oxalotrophic bacteria, which increases pH while releasing HCO3- and Ca2+. This phenomenon could have a global importance for the transfer of atmospheric carbon dioxide to calcium carbonate, acting as a stable carbon sink.

Previous studies have clearly demonstrated the importance of bacteria in this pathway and it is also well known that fungi play a role as producers of oxalate. More recently it has been revealed that fungi are also able to decay calcium oxalate, but almost nothing is known about their importance in oxalate turnover. Therefore, the aim of this study provides insight into bacteria – fungi interaction in the oxalate-carbonate pathway.

To study the impact of this interaction on calcium oxalate transformation to calcium carbonate, a microcosm experiment was set up. Sterilized, acidic, carbonate-free and straw amended soil with or without addition of calcium oxalate was inoculated by bacteria only, fungi only, or fungi and bacteria. Microcosms were monitored for three months, destroying one microcosm at each sampling time.

Although a part of the bacterial strains chosen for the inoculums are able to consume calcium oxalate both on solid or liquid media, it appears that in the microcosms the presence of fungi is essential to allow bacteria to degrade calcium oxalate and shift the pH (Fig. 1). Besides, the role of bacteria was confirmed by a strong correlation between pH shift and oxalate degradation. Furthermore the evolution of the frc gene abundance also highlights the importance of bacterial metabolism in the oxalate degradation. This metabolism seems to be unable to take place in a sterile oxalate amended soil without the presence of fungi. This point drives many questions about the mechanism that make this interaction so important.

Further study is necessary to verify if these types of interactions can be observed in a natural soil in order to discover the kinds of interactions taking place in our system.

Key words: bacteria; fungi; interactions; microcosms; oxalate; carbonate; carbon cycle; q-PCR; soil ecology

Figure 1: Evolution of pH during the experiment. B: microcosms (mc) without Ca-oxalate inoculated by bacteria only, F: mc without Ca-oxalate inoculated by fungi only, FB: mc without Ca-oxalate inoculated by fungi and bacteria, Box: mc with Ca-oxalate inoculated by bacteria only, Fox: mc with Ca-oxalate inoculated by fungi only, FBox: mc with Ca-oxalate inoculated by bacteria and fungi.
Gene expression during long term survival of *Escherichia coli* O157:H7 in soil and water

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The in vitro survival and growth of *Escherichia coli* O157:H7 under two experimental conditions (sterile soil and sterile natural water at 15°C) were examined over a period of several months. DNA microarrays of the entire set of *E. coli* O157:H7 genes were used to measure the genomic expression patterns of cells incubated under these conditions for 14 days. Although the population decreased by approximately 3.57 log, some *E. coli* O157:H7 cells survived in sterile stream water up to 254 days. Cells also survived in sterile soil for at least up to 179 days. Furthermore, it was found that 26 genes were more significantly expressed in cells grown in Luria broth at 15°C for 48 hours, whereas 12 genes were more significantly expressed in cells incubated in sterile stream water for 14 days at 15°C. The comparison of cells incubated in soil versus cells grown in Luria broth yielded more differences: 89 genes were expressed at significantly higher levels in Luria broth while 308 genes were expressed at significantly higher levels in cells incubated in sterile soil for 14 days at 15°C. An analysis of functional groups revealed that cells incubated in sterile soil microcosms expressed genes for antibiotic resistance, biosynthesis, DNA replication and modification, metabolism, plagues, transposons, plasmids, pathogenesis and virulence, ribosomal proteins, the stress response, transcription, translation, and transport and binding proteins at significantly higher levels than cells grown in Luria broth. These soil-incubated cells had a high level of expression for 18 genes that confer protection from environmental stresses as compared to cells grown in Luria broth. In addition, the soil-incubated cells had a high level of expression for three genes for antibiotic resistance and seven genes for pathogenesis and virulence. These results suggest that *E. coli* O157:H7 may develop a different phenotype during transport through the environment. Furthermore, this pathogen may become more resistant to antibiotics making subsequent infection with *E. coli* O157:H7 more difficult to treat.

Keywords microarrays; survival; *Escherichia coli* O157:H7

Haloalkaliphiles bacteria isolated from a saline soda environment from central Mexico

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Soda lakes and soda deserts are the most stable naturally occurring alkaline environments on Earth, where pH values of 10 and above are common. These environments are characterized by large amounts of sodium carbonate, or complexes of this salt, formed by evaporative concentration. Other salts, especially sodium chloride, lead to the formation of alkaline saline lakes which range from 5% w/v to 35% w/v (saturation) salts and have pH values from 8.5 to 11.5. The soda lake microbial community contains alkaliphilic and halophilic which are bacteria representatives of all the major trophic groups of extremophiles bacteria. Alkaliphiles consist of two main physiological groups of microorganisms; alkaliphiles and haloalkaliphiles. Alkaliphiles require a pH of 9 or more for their growth and have an optimal growth pH around 10; whereas haloalkaliphiles require both an alkaline pH (pH 9) and high salinity (up to 33% [wt/vol] NaCl). Haloalkaliphiles have been mainly found in extremely alkaline saline environments, such as the Rift Valley lakes of East Africa and the western soda lakes of the United States. Mexico is very rich in these types of extreme environments, and besides some reports on the haloalkaliphile Teococo Lake, little is known about the bacterial diversity found in the rest of the country. Currently a project is being carry out to found in Mexico environments where these bacteria growth. Specifically this work is focus in the studies carried out in San Luis Potosí.

The objective of this work was to assess the diversity of cultivable haloalkaliphile bacteria present in the saline soda environments of San Luis Potosí, Mexico. A polyphasic approach was chosen to characterize the isolates; morphological, physiological (salinity and pH tolerance), and nutritional (use of 40 C sources) features were used, in combination with 16S rRNA sequence analysis.

Samples were collected from a saturated hypersaline alkaline soil in San Luis Potosí, México, located at 22°37′39″N 101°42′52″O; this environment has high salt concentration (23% NaCl) and pH 10.

Seventeen strains were isolated in a modified HM medium. To identify the isolated strains, sequence of 16S rRNA genes was determined and compared whith sequences in the Gen Bank database using the Blast and EzTaxon programs.

Phylogenetic trees were built using sequences of 1500 base pairs including representative bacteria of halophilic species. The stability of each cluster was determined by bootstrap analysis of 1000 replicates.

We report the presence of the genera: *Gracillibacillus*, *Bacillus* and *Halomonas*. From the 17 isolates, seven strains were identified as *Gracillibacillus salophilus* and three as *Gracillibacillus orientalis*. Two strains were identified as *Bacillus mannanolyticus*, and four strains were identified as *Halomonas magadiensis*. They are able to grow from 5 to 15% NaCl, with an optimum at 10% NaCl. The range of pH for their growth is from 8-10, with the optimum at pH 9. The genera found in alkaline soil of San Luis Potosí, Mexico include halophilic and alkalophilic species that have been found in different geographical locations in the Earth, however this is the first report regarding the presence of these genera in Mexico.

Keywords: alkaliphiles; haloalkaliphiles; halophilic.
Hydrolytic biocatalysts isolated from intestinal microbiome in black soldier fly larvae, Hermetia illucens

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Black soldier fly, Hermetia illucens L., is a common colonizer of animal wastes. In its life cycle, larvae are voracious feeders of organic material, and may thus be used in simple engineering systems to convert large amounts of organic waste into protein-rich biomass. In an effort to identify contributing biocatalysts at the prepupal stage, we have constructed a metagenomic fosmid library of an average insert size of ~30kb using the larval intestinal microcosms. To detect hydrolytic enzymes encoded by uncultured intestinal microorganisms that help H. illucens for processing daily food wastes, individual 92,544 metagenomic clones were subjected to substrate hydrolysis analysis. Initial screenings of the libraries revealed 18 clones that use carboxymethyl cellulose as a sole carbon source. Additional screenings characterized four clones capable of starch degradation. Short-gun sequencing of the fosmid clones demonstrated putative cellulase and hydroxylase acting on cellulose or starch substrate. These results indicate that H. illucens hydrolyze manure nutrients in association with uncultured intestinal bacteria, enabling the fly to colonize successfully on a variety of animal wastes.

Keywords Hermetia illucens; Metagenome; Hydrolysis; Biocatalyst

Identification of hepatotoxic cyanobacteria in the environment

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Cyanobacterial mass occurrences (blooms) occur frequently worldwide and contain often hepatotoxic species, posing a risk for water users. The most common cyanobacterial hepatotoxins are microcystins and nodularins. They are cyclic peptides with seven and five amino acids, respectively, constructed by large peptide synthetase polyketide synthase enzyme complexes. Microcystins are produced mainly by strains of the genera Anabaena, Microcystis, and Planktothrix in fresh waters, while nodularins are solely produced by strains of Nodularia in brackish waters. Blooms can be comprised of several species, which may include potential hepatotoxin producers. However, toxic strains look alike non-toxic strains, and cannot be recognized by conventional microscopy. This makes it difficult to assess the risk for the water users. The presence of microcystin (mcy) or nodularin synthetase (nda) gene clusters encoding the biosynthetic enzymes is a prerequisite for toxin production and offers a practical way for differentiation and identification of hepatotoxin producers. Our aim was to develop methods based on the mcyE/ndaF genes to detect and identify hepatotoxin-producing cyanobacteria in environmental samples. The mcyE/ndaF genes encode enzymes involved in the synthesis of Adda and D-Glu, the constituent amino acids crucial for the toxicity of microcystin and nodularin molecules. In addition, the mcyE/ndaF genes are present in the biosynthetic gene clusters of different producer genera and thus allow detection of both microcystin and nodularin producers. Using conventional PCR and genus-specific primers we discovered that the majority of 70 Finnish lakes studied contained potentially microcystin-producing cyanobacteria, Microcystis being the most prevalent. In addition, the co-occurrence of potentially microcystin-producing Anabaena, Microcystis, and Planktothrix was common, especially in the more eutrophicated lakes. In a two-year survey of Lake Tuusulanjärvi, quantitative changes in the population of microcystin producers were followed by quantitative real-time PCR (qPCR) assays, detecting the mcyE genes of Microcystis and Anabaena.

In both years, Microcystis was detected almost throughout the year, while Anabaena became more common in late summer and fall. A DGGE analysis of a general mcyE/ndaF-PCR product revealed for the first time the widespread occurrence of microcystin-producing Anabaena in the Baltic Sea, where nodularin-producing Nodularia had previously been regarded as the sole hepatotoxin producer. As opposed to PCR methods, a DNA-chip using genus-specific probes for the mcyE/ndaF genes of Anabaena, Microcystis, Planktothrix, Nostoc, and Nodularia was designed to reveal all potential microcystin/nodularin producers simultaneously in a single analysis. In addition to specific and sensitive detection obtained with axenic cyanobacterial strains, the mcyE/ndaF genes were reliably identified in both lake and Baltic Sea samples. This makes the chip assay suitable for high-throughput analysis and monitoring of environmental samples. The chip assay and qPCR methods were further optimized to be used with environmental RNA as target. Detection of mcyE-mRNA implies active transcription of the biosynthetic gene and thus serves as a more reliable indication of actual microcystin production in the lake water samples.

Keywords cyanobacteria; hepatotoxin; microcystin/nodularin synthetase genes; molecular detection methods
Identification of morphological, biochemical and molecular markers for discriminating among Botryococcus braunii strains belonging to A and B chemical races

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Botryococcus braunii Kützing is a colonial green microalga, ubiquitous in fresh and salt waters from temperate and tropical regions. B. braunii is unique among the algae due to its ability to synthesize large quantities of hydrocarbons (up to 75% from dry biomass) very similar to the fossil oil. Chemical derivatives of hydrocarbon (botryococcus) and fossil records of the B. braunii colonies were identified in oil and boghead coal deposits from various geographical regions. B. braunii strains are classified into three chemical races based on produced hydrocarbons: the A race which produces linear and odd-numbered cis and trans (C15-C31) n-alkanes and -n-alkenones; the B race is the producer of triterpenoids called botryococcenes and squalene (C30-C32) of general formula C30H50 to the L race which synthesizes a tetrapentenoid called lycopadiene. Among the three, the B race seems to be the most promising as a source of liquid fuel because the content of hydrocarbon is generally higher than in other races, but the slow rate of growth (doubling time of 7 days) is the major inconvenient in this matter. Numerous attempts have been made to improve the growth process and to corroborate it with hydrocarbon synthesis using different combinations of growth parameters. A selected time of approximately 3 days was the best obtained in air (1% CO2) and the permanent stirring conditions, a much lower value with respect to other green algae (Chlorella spp., Scenedesmus spp.). Morphological, biochemical and physiological characterization of a higher number of strains, collected from all over the world could be a possible answer in this matter. Subsequently, this paper focused on: a) culturing of approximately 30 strains from A and B race collected from different areas of Transylvania region (Romania); these strains were included in the Algal and Cyanobacterial Culture Collection of the Institute of Biological Research (AICB), Cluj-Napoca, Romania; b) morphological characterization of both A and B races emphasizing their specific features; c) characterization of growth process of certain strains cultivated in a bioreactor with or without CO2 supplementation; d) biochemical description of strains, based on produced hydrocarbons and fatty acids; e) phylogenetic analysis based on rDNA sequences; f) establishing a possible relationship between morphological, biochemical and molecular features in order to discriminate between the two races. Different methods and techniques were used: the isolation by micromanipulation of different strains and their cultivation in BG 11 medium; light and electron microscopy (SEM) analysis; gas chromatography-mass spectrometry coupling (GC-MS) protocols for quantitative and qualitative characterization of hydrocarbons and fatty acids; isolation and amplification of rDNA fragments by PCR, using 5 specific primers; sequencing and phylogenetic analysis using various computational programs (for sequence identification and alignment, Vector NTI); alignment and phylogenetic trees construction (Mega 4.1). The specific morphological features and hydrocarbon pattern identified in this study sustain the B. braunii strains classification in the A and B races. Thus, morphologically, the strains belonging to the A race possess green colonies with a smaller number of cells, with only the basal part embedded in the colonial matrix. The cell apex is always exposed and in some cases (AICB 851 strain) a transparent "cap" could be observed. Numerous singular cells or small clusters of 2-6 cells have been observed in suspension. The hydrocarbons were present mostly as lipid bodies inside the cells. Compact colonies were observed in the strains of B race, with a greater number of matrix. The cells were almost entirely embedded in the matrix, the cell apex being rarely exposed. Biochemical analyses highlight the presence of C29 alkadiene in all 30 strains, whereas just half of them contained C31 alkadiene. Only 4 strains belonging to A race contained C29 alkadiene. Almost all investigated strains contained C30-alkadiene. The C30-C32 botryococcenes represented the bulk fraction of total hydrocarbons in the strains belonging to the B race, but traces of these were identified in some strains from A race, also. The total hydrocarbon content (% from dry biomass) ranges between 0.031 and 16.45%, for the A race and 8.5-36.45% for the B race, respectively. Differences in hydrocarbon content correlated with growth conditions have been noticed. The unsaturated fatty acids prevailed in all strains, whereas linoleic, palmitic and oleic fatty acids were dominant. Based on partial rDNA sequences, all the strains investigated belonging to the AICB collection form a tight monophyletic cluster with other B. braunii strains derived from public sequence databases.

Keywords: Botryococcus braunii, morphology, hydrocarbons, phylogeny.

Identification of toxic cyanobacteria from environmental samples based on PCR amplification of a mcyD gene fragment

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Introduction Cyanobacteria are prokaryotic organisms well-known for their capacity to produce cyanotoxins, some of them known as microcystins. These are various secondary metabolites of peptide nature, nonribosomally synthesized by a multi-enzymatic complex consisting in peptide synthetases and poliketide synthases. As heptapeptides, microcystins are very small molecules, and their identification or characterization is very difficult to achieve, and this is the reason why the cyanobacterial blooms may be extremely dangerous. The chemical cell lysis is not a proper key in reducing the intoxication risk, because in this way the toxins are released in the environment. The most suitable solution to this problem is to find a way to identify the toxic cyanobacterial blooms during their initial development phases, in order to prevent the risk of intoxication. The purpose of this study was to find proper oligonucleotide primers able to provide a reliable and quick detection of the toxic cyanobacteria from environmental samples.

Methods The 24 cyanobacterial strains used in this work belong to genus Microcystis, and they are original isolates from Romanian waters, provided by The Cyanobacteria and Algae Culture Collection (AICB) of the Institute of Biological Research, Cluj-Napoca, Romania. The hepatotoxic potential of the strains was analyzed by PCR amplification of certain genomic regions responsible for the synthesis of the enzymes involved in toxin production. An additional experimental assay was represented by MALDI-TOF MS (Matrix Assisted Laser Desorption /ionization: Time Of Flight Mass Spectrometry) technique. In order to achieve a correlation between PCR and MALDI-TOF test we tried to discriminate the ampiclons by DDGE (Denaturing Gradient Gel Electrophoresis). Eventually, we have sequenced the PCR amplicons (BigDye terminator method) and we have designed new primer pairs able to discriminate between toxic / non-toxic cyanobacterial strains. We have also verified the quality of the primers on 4 environmental samples, the results being also certified by additional MALDI-TOF analyses.

Results The MALDI-TOF MS spectra have displayed the presence of microcystins in 5 of the 24 cyanobacterial strains: Botryococcus, microcystin and other related secondary metabolites being also detected. The PCR amplification with primers known from literature did not allow the detection of microcystins entirely correlated with the toxicity tests. The DDGE profiles could not discriminate between the generated amplicons according to the strains toxicity. Multiple alignments of the obtained sequences allowed us to design an improved primer pair, which was specific for the 5 toxic strains. The primers also allowed us to identify toxic cyanobacteria from the 4 environmental samples, the MALDI-TOF spectra confirming the presence of toxins.

Conclusions We have observed that the oligonucleotide primers found in literature did not generate PCR amplicons in our toxic strains only, as expected, but also in other two non-toxic strains. The DDGE technique was not able to distinguish the amplicons according to strains toxicity. Based on the sequenced amplicons and their multiple alignments, we have obtained a new primer pair which is able to amplify a certain region of the gene cluster responsible for toxicity, this being highly specific for our toxic strains. The most important result is that the new PCR primers are able to identify microcystins from environmental samples, which is a very important part for an early detection of a putative toxic cyanobacterial bloom.

Keywords: Cyanobacteria, blooms, microcystins, MALDI-TOF MS, PCR
Impact of copper on denitrification process and on the microbial communities involved.

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Denitrification influences the nitrogen budget in estuaries by removing fixed nitrogen from the inorganic pool; rates are dependent on both geological and geographic conditions as well as increasingly anthropogenic impacts. In this study, the effects of copper (Cu), on the denitrification pathway was evaluated in intertidal sandy sediments of the Douro River estuary. Dinitrogen (N₂), nitrous oxide (N₂O), nitrite (NO₂⁻) and nitrate (NO₃⁻) production rates were measured in triplicate slurries of field samples under different treatments of Cu concentrations. In addition to activity measurements, the diversity of genotypes and transcripts of nitrite reductase genes (nirS and nirK) was evaluated by DGGE (denaturing gradient gel electrophoresis) and the respective semi-quantitative and quantitative analysis performed by real time PCR. Results demonstrated that denitrification rates in intertidal sediments of the Douro River were highly affected by Cu. An almost complete inhibition of denitrification by Cu (85%) was observed in sediments amended with 79 μg per gram of wet sediment. Moreover, the addition of Cu stimulated N₂O (a powerful greenhouse gas) and NO₂⁻ (a potential toxic compound) accumulation in intertidal sandy sediments, demonstrating a pronounced inhibitory effect on specific steps within the denitrification enzymatic system. In agreement, semi-quantitative and quantitative PCR of denitrification genes and transcripts revealed lower nirS and nirK transcription within the treatments with higher concentration of Cu, while DGGE analyses showed no clear changes in the denitrifier community structure within the different treatments performed.

Impacts of Metal Nanoparticles on Leaf Litter Decomposition by Aquatic Fungi

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Worldwide industrial scale production and commercialization of engineered nanoparticle (NP) based products increases the probability of their release into freshwater environments raising the question of whether NPs pose a risk aquatic biota and the associated ecological processes. In streams, aquatic hyphomycetes are a group of fungi that play a key role in organic matter turnover serving as intermediaries between plant litter and higher trophic levels. In this study, the effects of copper and silver NPs on leaf litter decomposition by aquatic fungi were examined and results were compared with those of their metal sonic forms. Leaves of Alnus glutinosa were immersed for 7 days in the Maceira stream (Peneda-Gerês National Park, Northwest Portugal) to allow microbial colonization, before exposure in microcosms to different nominal concentrations of the two metal NPs (CuO, 100, 200 and 500 ppm; Ag, 100 and 300 ppm) and metal ions (CuCl₂, 10, 20 and 30 ppm; AgNO₃, 5 and 20 ppm) for 21 days at 13 °C. In non-exposed microcosms, a total of 11 aquatic hyphomycete species were identified based on spore morphology, among which Articulospora tetracladia (51.2%) and Flagellospora sp. (32.8%) were dominant on decomposing leaves. The exposure to metal NPs or metal ions decreased the number of sporulating fungal species (4 – 10 species in the treatments with 30 ppm CuCl₂ and 100 ppm Ag NPs, respectively). At the highest stress levels, the contribution of A. tetracladia (55.5 – 76.1%) and Heilsicus lugdensis (8.1 – 22.6%) to the total spore production increased, while that of Flagellospora sp. (6.5 – 15.9%) decreased. At the end of experiment, metal NPs significantly reduced fungal biomass (28.5 – 82.9% inhibition in treatments with 100 ppm Ag NPs and 30 ppm CuCl₂, respectively) and reproduction (90.9 – 99.4% inhibition in treatments with 100 ppm Ag NPs and 30 ppm CuCl₂, respectively), probably explaining the reduction in leaf mass loss. Moreover, the negative effects of metal ions on leaf decomposition by aquatic fungi were more pronounced compared to their nanoparticle forms, despite metal ions were applied at one order of magnitude lower concentrations. Results indicate that high concentrations of metal NPs may affect aquatic fungal communities with impacts on leaf litter decomposition in streams.

Keywords: metal nanoparticles; metal ions; copper; silver; streams; microcosms; leaf decomposition; aquatic hyphomycetes
**In vitro methane reduction through bacteriocins of rumen isolates**

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Methane emission in environment poses a challenge to the agricultural scientists, as ruminants immensely contribute to it, while resulting in 6-15% of energy loss to the animal, thereby affecting productivity. The present study deals with the screening of bacteriocin producing potential of rumen bacteria isolates and effect of their bacteriocin on rumen methanogenesis. For this, a total of 10 rumen samples were taken form fistulated buffaloes and screened for their bacteriocin producing ability by deferred antagonistic assay. Out of the 28 isolates tested, only two isolates i.e. BR8 and BR15 were found to be bacteriocin producers. The crude extracts of bacteriocins from both the cultures were used along with diet containing high roughage ratio (80%) to assess their effect on methanogenesis. Methane was analyzed through gas chromatography and no effect of BR15 extract in methane reduction (% methane in ml) was found, whereas 10 times diluted extract of BR8 showed a reduction in methane from 4.5 to 2.8%, indicating a positive effect of bacteriocin in methane reduction. The isolate BR8 was subjected to identification through physiological and biochemical tests, followed by 16S rDNA based PCR identification and identified as *Streptococcus bovis*.

**Key Words:** Rumen; Methane; Bacteriocin

**INDISIM-SOM: an individual-based simulator on a website for experimenting and investigating diverse dynamics of Carbon and Nitrogen in mineral soils**

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A very interesting review of soil biogeochemical models, with an extensive comparison of mathematical approaches to soil C and N cycling, has been provided by Manzoni and Porporato [2]. Some key processes in C and N cycling in soils are the decomposition and mineralization of organic matter (OM), N immobilization and nitrification. These processes, in the majority of models developed during the last decades, are analyzed under the common framework of substrate-decomposer stoichiometry, thus stressing the role of the microbial biomass as a Soil OM (SOM) degrading agent and as a controlling factor of N cycling. Most new models are improvements over earlier ones, leading to many similar model structures and formulations. While this has generally produced more robust and effective models, on the other hand, it may have delayed significant theoretical advances and shifted attention from some important questions that have therefore remained unexplored. There are fewer models explicitly describing the spatial dynamics of water, OM or nutrients at specific microscopic scales [2]. Some researchers are realizing that simulation models are a new kind of experimental system. Individual-based models (IBMs), in which individuals interact dynamically with each other as structural elements in the model world, exemplify this view of simulation modelling. In this context, the present study is based on the perspective that a mechanistic and scale-dependent description of microbial activity, with detailed formulations of decomposer biomasses and their relationships with organic and mineral substrates, is essential when dealing with the dynamics of C and N in SOM. Nevertheless, the level of microbial activity, and not only the amount of microbial biomass, is fundamental to describe transient fluxes in response to environmental fluctuations. To provide the generality needed for diverse scenarios, under different conditions, one way to proceed is by using IBMs, from which macroscopic patterns may be inferred. INDISIM-SOM models the dynamics and evolution of C and N related to SOM by using individual-based simulations [1]. It is an IBM that controls a group of microbial cells at each time step, using a set of time-dependent variables for each microorganism. The space is divided into square cells. In each spatial cell, the amounts of different types of organic compounds are controlled. These are identified as polymerized organic C and N, labile organic C and N, and mineral compounds like ammonium $N_{NH4}$, CO$_2$ and O$_2$. The model takes into account the activity of two types of microorganisms: decomposers (heterotrophs) and nitrifiers. Metabolic pathways and sources of C and N that they can use are identified. Some state variables and parameters related to SOM and microbial activity are studied [1]. The purpose of this work is to develop and present a website from which the simulator INDISIM-SOM is accessible, and to explain how to carry out some virtual experiments, in order to further advance the skills associated with this simulation model. The website is composed of the following: i) a brief theoretical introduction of the general model, ii) a demonstration of the simulator with graphical outputs for some variables (Demo option) and iii) access to an executable version of the simulator allowing changes in the values of some parameters (Log in option). The input data offered for modification, jointly with graphical outputs, make it possible to configure virtual experiments and observe their behaviour through the simulator. The parameters which can be changed before starting the simulation are related to i) soil properties, ii) fractionation constants, and iii) microbial characteristics. The first one is related to soil determinations of C and N, and the second one is made up of the constants that relate the soil properties to discrete soil compartments. The last consists of data related to soil microorganisms, and related output variables: number of heterotrophic individuals, number of nitrifier bacteria, ratio of microbial C to organic C, ratio of migrating N under different C/N microbial ratio and oxygen in soil atmosphere. The other set of output graphics is: net production of CO$_2$, easily hydrolysable N, net N mineralization, total ammonium in the medium, and nitrate in the soil solution. The graphical output shown allows the user to visualize how system variables emerge from different scenarios. This web application results in a very versatile program that could be used in controlled simulation experiments via Internet, and it is a useful way to analyze the INDISIM-SOM simulator in order to achieve further understanding of soil system modelling.

**Keywords** Soil Organic Matter, dynamics of Carbon and Nitrogen, Individual-based Modelling

Influence of Lead Contamination on Bacterial Community in Pine Forest Soil
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Hälvälä, a shooting range in pine forest located in southern Finland, is heavily contaminated by Pb. To study the effect of Pb contamination on bacterial community, the range was divided into clean area (C) and Pb contaminated area (P) according to the Pb concentration. Bacterial diversity maps were generated by means of DNA based molecular biotechnologies in both clean and Pb contaminated pine forest soil. 918 sequences of the beginning part of 16s rRNA genes (about 400 bps) were achieved from the two study areas (C: 430, P: 488). 399 OTUs were found by grouping similar sequences at the similarity rate of ≥97%. There were 213 and 253 OTUs in clean and Pb contaminated areas, respectively. In each area, Proteobacteria that takes about 40% sequence frequency is the biggest group, the second largest group is Acidobacteria that occupies approximately 35% and 10% of Actinobacteria is found. Other bacterial phyla such as Verrucomicrobia, Bacteroidetes, Planctomycetes and Firmicutes are spotted but considered as minor groups, since total frequency of them in each area is less than 5%. In general, no clear difference of bacterial diversity is found between clean and Pb contaminated areas at phylum level.

Keywords heavy metal; bacterial diversity

Influence of the fungicide Benomyl and ecophysiological factors on growth rate of the fumonisins-producing Fusarium verticillioides and Fusarium proliferatum from Spanish maize
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Fusarium verticillioides and Fusarium proliferatum are considered the most important maize fungal pathogens causing Maize Pink-Ear Rot or Maize Saturt Rot and both also have the ability to produce fumonisins. Fumonisins are one of the most important mycotoxigenic compounds responsible of both human and animal toxicoses caused by consumption of contaminated food and feeds, especially corn-based commodities. The occurrence of fungal species and their toxin production is influenced by ecophysiological factors such as solute stress or temperature, and also depends on interactions with other species. Currently, control of fungal disease relies almost exclusively on the application of antifungal agents, particularly when weather conditions are conducive to infection (from anthesis to harvest). Although there are a number of compounds with in vitro activity against fumonisin-producing Fusarium pathogens, little information is published on the interaction of these compounds with the ecophysiological factors, which might influence the efficiency of these compounds to control fungal growth. The objective of this work was to study the impact of the commonly used fungicide benomyl (LD₅₀ and LD₉₀) on growth rate of F. verticillioides and F. proliferatum isolated from maize in Spain and the interaction with the main ecophysiological factors influencing fungal growth (water activity and temperature). The growth rate of the two isolates was evaluated in relation to water activity (a_w: 0.995, 0.98 and 0.95) and temperature (20, 25, 30 and 35°C) on a solid fumonisin producing medium (in vitro). In general, the fungicide Benomyl reduced growth rate when compared to the control (LD₅₀) in all a_w values considered, and this reduction increased with increasing fungicide concentration, except at 0.95 a_w, in both species. At 0.95 a_w, growth rate of LD₅₀ and LD₉₀ had similar profile in both species and only at LD₅₀ a slight reduction of growth could be observed, indicating that the antifungal activity is not efficient at higher water stress values. Both species also showed similar pattern in relation with temperature, although F. proliferatum showed a more retarded pattern of growth rate. The similar behaviour of these species against the benomyl might represent an advantage when a treatment with this fungicide must be applied since both are often present in maize. Those data are important to enable more efficient control strategies during the life cycle of these important maize mycotoxigenic fungal pathogens.

Keywords: Fungicide; growth rate; F. verticillioides; F. proliferatum.
Influence of the heavy metals on chitosan production by *Absidia corymbifera* UCP 0134

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This work describes the experimental studies on the influence of the addition of the heavy metals copper (Cu) and zinc (Zn) in different conditions, on the fermentation of *Absidia corymbifera* on cultivation containing corn steep liquor 6% (residue of the corn industry), and the heavy metals were added in the concentrations of 2 and 4 mM. The experiments were incubated at orbital shaker at 150 rpm, temperature of 28°C for 96 hours. The biomass was removed by vacuum filtration, lyophilized and maintained in dissector until constant weight, evaluated by gravimetry. The polysaccharides chitin and chitosan were extracted by alkali, acid treatment. The polysaccharides were characterized by Transformed of Fourier- Infrared Spectroscopy (FTIR). The higher yield of the biomass was obtained in the condition copper 4mM – (6.97g/L). The highest chitosan production was obtained in the conditions zinc 4mM (44.5mg/g of biomass), and copper 2mM (67.29mg/g of biomass). The acetylation degrees was 81 to 88%.

These results suggest that the addition of heavy metals copper and zinc to the culture medium promoted an increase in the production and enzymatic action of chitin synthetase and chitin deacetylase, involved in production processes chitosan by *Absidia corymbifera*. The low cost medium used in this experiment to make large production viable for future laboratorial and industrial assays.

**Keywords:** Chitosan, Heavy metals, *A. corymbifera*.

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Inhibition Effect of Isopropanol on Expression Level of Acetyl-CoA Synthase Gene in Anaerobic Processes

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Organic solvents which are found in several industrial waste streams may cause inhibitory effects on microbial community playing role in anaerobic processes. Effects of the compounds on different metabolic steps are largely unknown. Since aceticlastic methanogens participating in the terminal methanogenesis step play an important role in stabilizing the pollution load of wastewater and susceptible to inhibitory compounds, understanding the specific inhibition effects of the pollutants on aceticlastic pathway in anaerobic reactors is important. Although there are some studies in the literature on treatability of the solvents under anaerobic conditions in which different electron acceptors are present, specific studies that reveal the effects of the compounds at different conditions on key metabolic steps are limited. Therefore, in this study, inhibition effect of isopropanol on both biogas production and expression level of acetyl-coA synthase of Methanosaeta, which is a key enzyme of methane production from acetate in the anaerobic treatment processes, were monitored. For this purpose, an anaerobic sludge with concentration of 2000 mg/L was added to the 100 ml serum bottles with a dilution solution. Anaerobic conditions were provided by flushing the bottles with nitrogen gas. Sludge was incubated at 37°C for 24 hours and then was fed with acetate with a concentration 2000 mg/L. After the incubation period, the anaerobic sludge was fed with acetate and isopropanol at different concentrations in between 0.1 M-1.0 M and the bottles were kept in a stirred water bath at 37°C for 7-10 days. Batch serum tests were exposed to isopropanol at different concentrations for three times. Methane composition was monitored regularly and next exposure was performed when the gas composition was stabilized in serum bottles. Before each exposure, samples were taken from the reactors and, then, the effects of the solvents on the expression level of acetyl-coA synthase was measured by quantification of mRNAs using Q-PCR. A new designed primer pair which is specific for acetyl-coA synthase gene was used for the purpose. After second exposure, numbers of the gene copies per ml were found to be 1.5E+06, 0.56E+06, 0.41E+06, for 0.1M, 0.5 M and 1.0 M isopropanol concentrations respectively whereas that of was 1.66E+06 for control reactor fed by only acetate. No genes can be quantified after third exposure due to severe inhibition effect of the solvent. Methane composition and gas pressure also decreased with increasing isopropanol concentrations and repeated exposures (Table 1). The decrease was dramatic especially for third exposure indicating that repeated exposure of isopropanol to aceticlastic methanogens can cause severe inhibition even for low concentrations of the solvent.

<table>
<thead>
<tr>
<th>Isopropanol (M)</th>
<th>1st Exposure</th>
<th>2nd Exposure</th>
<th>3rd Exposure</th>
<th>2nd Exposure</th>
<th>3rd Exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 M</td>
<td>94</td>
<td>84</td>
<td>24</td>
<td>1905</td>
<td>595</td>
</tr>
<tr>
<td>0.5 M</td>
<td>84</td>
<td>65</td>
<td>12</td>
<td>1460</td>
<td>235</td>
</tr>
<tr>
<td>1.0 M</td>
<td>75</td>
<td>36</td>
<td>3</td>
<td>435</td>
<td>240</td>
</tr>
</tbody>
</table>

**Keywords:** contamination, gum production, pencillin, xanthan, Xanthomonas campestris.
Isolation and Characterization of Atrazine and Terbutryn Degrading Microbes from Surface and Subsurface Soil in Finland

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The extensive use of triazines atrazine and terbutryn has resulted in groundwater contamination in many areas, including some groundwater sources in Finland. In soil surface, atrazine and terbutryn are degraded in a few months by microorganisms. However, the degradation rate of microbes often decreases with increasing the depth in soil, and some of pesticides are stored at deep sediments for years. We isolated atrazine or terbutryn degrading microbes from enrichment cultures using selective medium, which have atrazine or terbutryn as a sole nitrogen source. Enrichment cultures originated from surface soil and groundwater sampling pipe deposits. In addition, some indoor isolates were obtained. The strains were characterized using ribosomal DNA sequencing and fatty acid analyses. Altogether 110 microbial strains degrading atrazine or terbutryn were isolated. They belonged to 19 different genera, including Acinetobacter, Acremonium, Alphaproteobacteria, Aspergillus, Bacillus, Bradyrhizobium, Burkholderia, Janthinobacterium, Methylobacterium, Microbacterium, Penicillium, Ralstonia, Rhodococcus, Sphingomonas, Streptomyces, Variovorax, and Williamsi. The results on the phylogenetic diversity of atrazine or terbutryn degraders in these different environments were elucidated. The differences in microbial profiles may be of importance in use of strains in remediation of contaminated soils and groundwater environment.

Keywords: atrazine; microbial degradation

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Isolation of an antibiotic producing bacterium from the Persian Gulf

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During two previous decades, studying about marine microorganisms has been raised significantly due to their very high capability of producing secondary metabolites. In fact, the strong selective pressure result from competition between bacteria in the marine environment for space and nutrition and also metabolic and physiological differences between marine and terrestrial microorganisms arising from different conditions in marine would result in these microorganisms produce a great amount of natural products for use in medical and industrial applications. Emerging pathogenic bacteria resistant to current antibiotics particularly MRSA (Methicillin Resistant Staphylococcus Aureus) strain has become a great global concern in 21st century. The aim of this study was to evaluate marine bacteria of different origins (water and sediment) from Persian Gulf in order to produce antibiotic compounds against some human pathogenic bacteria.

Initially we collected samples from surface seawater, deep seawater, coastal seawater, marine bed sediment and mangrove forest sediment at 18 study sites in the some northern areas of Persian Gulf including Mahshahr port, Bahranport and Qeshm Island. Marine Agar 2216 and Cellulolytic Agar with Sea Salt were used as primary isolation media. Pure colonies of isolated bacterial colonies were transferred to Erlenmeyer flasks containing Marine Broth medium and were incubated at 28º C on a rotatory shaker (150 rpm) to produce secondary metabolites. After 2 to 9 days, culture medium within Erlenmeyer was centrifuged at 10000 rpm for 20 minutes, and then supernatant was extracted by ethyl acetate. The antibacterial activity of the obtained raw extract was assessed at 100 mg/mL concentration by using disc diffusion method against pathogenic bacteria including MRSA, Staphylococcus epidermidis, Bacillus cereus, Bacillus pumilus, Listeria monocytogenes, Streptococcus pyogenes, Pseudomonas aeruginosa, Escherichia coli, Proteus mirabilis, Salmonella Typhi and Klebsiella pneumoniae. Five different antibiotics including Nafcillin, Vancomycin, Colistin, Methicillin, Oxacillin were used as control. Altogether, 42 bacterial isolates were obtained and only one brown-pigmented bacterium isolated from a marine sediment sample collected at a depth of 10 m was exhibited the capability of producing antibiotic compounds. On the 22 and 30 mm, respectively. Also, the best antibacterial effect of the obtained extract from Pseudomonas sp. PG-01 especially against MRSA, it can regard the intended bacterium as a valuable strain and further studies should be performed in order to purify and identify the chemical structure of its antimicrobial compound.

Keywords: antibiotic, Pseudomonas, resistance, MRSA, Persian Gulf.
Isolation of bacteriophage-host pairs from marine sediments

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Bacterial viruses (bacteriophages) are the most abundant and genetically diverse biological objects in the marine environment. It is estimated that there are about 10^{10} viral particles per liter of sea water and 10^9-10^{13} particles per kg of marine sediment (10-25 more than bacterial cells).

We have developed a high-throughput method of isolation of phage-host pairs from the sea-bottom sediment. First, we have isolated several hundred bacterial strains by plating on standard media used in marine microbiology (Zobell, Marine Broth, BOSS) at different temperatures (4°C, 22°C, 30°C). These strains were then used in phage isolation experiments, resulting in a collection of about 50 viral strains infecting bacteria found to belong mostly to *Pseudomonas* and *Shewanella* using 16S rRNA sequencing. Some of the isolated phages were found to have a wide host range and to tolerate a wide spectrum of temperatures (0°C – 37°C) and salinity (0%-5%). Virion morphology was analysed using electron microscopy; most isolated viruses had icosahedral heads and could be classified as *Myoviridae*. Complete sequencing of the bacteriophage genomes is now underway. Although the isolation method restricts the spectrum of investigated phages to the cultivable subpopulation, it has the advantage of allowing for the potential of investigating phages that have not yet been isolated.

**Keywords:** marine bacteriology, marine virology, bacteriophages, phage morphology, phage physiology, phage genomics.

Isolation of *Dunaliella salina* (Volvocales, Chlorophyta) and its growth characteristics

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The microalgae, *Dunaliella salina* (Dunal) Teod. maintained in De Walne’s medium was confirmed by amplifying the chromosomal DNA by PCR with specific primers MA1 and MA2. Seaweed extracts *Sargassum wightii* and *Ulva lactuca* were amended separately at 1.0%, 1.5%, 2.0% and 2.5% levels to the basal medium in order to assess their potential on the growth and concentration of pigments, viz. Chl a, Chl b and *β*-carotene at 9%–5%. Virion morphology was analysed using electron microscopy; most isolated viruses had icosahedral heads and could be classified as *Myoviridae*. Complete sequencing of the bacteriophage genomes is now underway. Although the isolation method restricts the spectrum of investigated phages to the cultivable subpopulation, it has the advantage of allowing for the potential of investigating phages that have not yet been isolated.

**Keywords:** marine bacteriology, marine virology, bacteriophages, phage morphology, phage physiology, phage genomics.
Isolation of high performance indigenous microbial consortia from highly acidic acid mine drainages contaminated soils

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Acid mine drainages (AMD) are contaminated water bodies characterized by high concentrations of metals and sulphates and by very low pH (1.5 to 3.5). In Portugal several abandoned mines produce AMD in amounts susceptible of creating ecological problems (contamination of aquatic flora and fauna and the surrounding soils. Although bioremediation has been considered a promising technology for AMD decontamination sulphate reduction rates measured in waters subjected to bioremediation are usually low. One of the main inhibitory factors is the toxicity of the heavy metals present in AMD. Therefore several attempts have been made to isolate indigenous microbial sulphate-reducing consortia from S. Domingos mines, one of the most contaminated sites in Portugal. The rationale for this study has been that sulphate-reducing bacteria isolated from AMD contaminated sites might be naturally tolerant to the AMD extreme conditions. Now, for the first time, we have isolated two sulphate reducing microbial consortia from the highly acidic soil (pH of 3.6) on the shore of the creek receiving the S. Domingos mine AMD waters. These natural consortia displayed very high rates of both sulphate and metal removal. After an adaptation period of 14 days, the consortium reduced sulphates at high rates (over 90%, from 3500 mg/L to 300 mg/L in 14 days) and displayed multiple tolerance to iron, copper and zinc, in concentrations equivalent to those present in the acid mine drainage. Moreover, the consortium removed iron by 99% (from 367 mg/L to 2 mg/L), copper by 80% (from 70 mg/L to 14 mg/L) and zinc by 96% (from 126 mg/L to 5 mg/L) within two weeks. The remarkable feature of these sulphate-reducing consortia is their very acidic natural habitat and their high sulphate and metals removal rates. In the following experiments, the bioremediation potential of the isolated consortia will be tested in artificial and natural AMD. Finally, the populations directly involved in the bioremediation process will be identified by molecular biology methods.
Isolation, Identification and Comparison of Cyanobacteria from Two Rivers Polluted with Different Chemicals

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In our study, water samples obtained from Porsuk and Seydisuyu Rivers located in Eskisehir-Kütahya Provinces of Turkey were used for cyanobacteria isolation. The compositions of pollutions in both rivers are quite different. Microscopic examination of water samples were made by using Olympus microscope. Cultivation of water samples were carried out with BG-11 and BG-11 medium at 28°C under the continuous light. Chemical properties of water samples were determined as spectrophotometrically using Merck test kits. After the visible growth in liquid BG-11 media, purification steps were performed with repeated plating techniques. At the end of this purification steps totally 127 pure cyanobacterial isolates were obtained from both rivers. For the identification of the isolates molecular analysis based on 16S rRNA gen sequences were performed. First step was genomic DNA isolation and then they were grouped according to their restriction enzyme profiles (Amplified Ribosomal DNA Restriction Analysis ARDRA). With the ARDRA analysis, one to two representative isolates were chosen and 16S rRNA gen was partially sequenced.

Based on the BLAST database, the sequences obtained from Porsuk River were Synechococcus, Cyanobium, Anabaenopsis, Leptolyngbya, Microcoleus, Pseudanabaena, Synechocystis, Oscillatoria, Snowella, Nostoc, Phormidium, and Limnothrix and Pseudoanabena genera and the sequences from Seydisuyu River were Leptolyngbya, Synechocystis, Phormidium, Anabaenopsis, Nostoc.

Joint culture of two defined methylotrophic strains for denitrification of ground water with natural gas as carbon source

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Ground water continues to be a major source of water in many cities. Water supply in most of these cities is highly contaminated with nitrate ion. Tehran and Mashhad water supply, two most populated cities of Iran, have highest level of nitrate in country, which has exceeded the drinking water quality standards. Since high nitrate levels in drinking water can cause serious human health effect then the United States Environmental Protection Agency (EPA) sets the maximum allowable level of nitrate-nitrogen in public drinking water at 10 milligrams per liter (10 parts per million).

When faced with the possibility of higher nitrate levels than allowed, water suppliers take some action like dilution of contaminated source with another source with lower amounts of nitrate or some treatments like reverse osmosis, ion exchange and biological treatment. Among these methods biological treatment has some advantages over others such as, 1) being relatively inexpensive and 2) having the ability to completely destroy the contaminants rather than producing a waste stream, which physical or chemical treatment methods tend to do. However, biological treatment of drinking water is challenging because the raw water generally does not contain enough organics to support a microbial population, which means a substrate must be added.

A variety of carbon sources, such as methanol, ethanol, acetate and sugars has been used as exogenous carbon source. The possibility to use methane as a substrate in the form of natural gas has some advantages over other carbon sources, such as, 1) natural gas is available at low cost and high amounts especially in Iran because of its broad gas reserves, 2) it spreads very fast in aquifer, 3) it is metabolized slowly, and at last 4) it can easily exit water phase. Then, the use of methane minimizes increasing in TDS of water. Since none of the known methanotrophs is able of denitrification, due to aerobic nature of methane oxidizers, then a bacterial consortium is necessary to use methane as the sole carbon source, in which the methanotroph may consume methane and release soluble organics that can be used by denitrifiers as electron donors in denitrification.

In this study, Methylobacterium extorquens DSMZ 1340 was used as the methanotrophic strain and Hyphomicrobium denitrificans DSMZ 1869 as the methylotrophic denitrifier because of their complementary properties, in other words, when H. denitrificans faces oxygen-starving conditions does not compete with M. extorquens in oxygen consumption and its nitrate respiration cycle will be activated. On the other hand, both of these strains were selected from risk group one and with some simple downstream treatments quality of water can be satisfied. The bioreactor setup was consisted of a bubble column with two separate methane and air inlets for prevention of probable explosion and a jacket to keep growth media in constant temperature of 30 °C with a water bath. Gas flow rates were controlled with flow meters. Inoculum was prepared for the two bacteria separately with methanol as the sole carbon source. Methane and airflow rates were constant during the process. Variations in nitrate concentration, optical density, dissolved oxygen, and oxidation-reduction potential (ORP) were analyzed and recorded. Insulation volume was about 10% (5 % from each) of bioreactor working volume (250 mL). Nitrate concentration at the beginning of the experiment was set to 100 ppm in the growth media.

Results presented good compatibility between these two bacterial strains for denitrification. In spite of the fact that this denitrification process was conducted by haphazardly selected variables, like air or methane flow rates, 54 % reduction for nitrate was achieved after 3 days. Higher amounts of nitrate reduction may be attained after the optimization of process variables involved in denitrification. ORP at the end of the process was about +90 mV, which is in the aerobic range. OD of 600 nm reached the maximum of 0.74 from 0.17 at the beginning. Selected flow rates for air and methane resulted in a DO of about 80 % of saturation.

keywords: Nitrate Removal; Natural Gas; Methanotrophic Bacteria; Methanotrophic Bacteria; Bubble Column
Methane production and oxidation in immobilized activated sludge from aerobic wastewater treatment plant

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The object of this study was the investigation of microbial community of immobilized activated sludge from the experimental aerobic wastewater treatment plant. The plant is situated in the settlement Krasnaya polyana (Sochi region), where the 2014 Winter Olympics is located. The main technique used is an original support material. It is similar to cleaning brush in shape and made of flexible polymeric fibers of varying thickness. The fiber is used for immobilization of microorganisms by means of which biological wastewater treatment takes place. The activity of microbial community being immobilized to brush-like flexible polymer support material was high. The activated sludge was demonstrated to contain aerobic and anaerobic microorganisms, inclusive of methanogenic archaea. The functioning of methanogenic microbial community, responsible for complete degradation of organic matter under anaerobic condition was shown. Generated methane was shown to be oxidized by methanotrophic bacteria, thereby methane cycle was realized. Meanwhile, methane generation occurs chiefly in immobilized form of active sludge and methane oxidation is typical for free-floating form. Volatile fatty acids, being generated as mediate products, were shown to be decomposed by aerobic and anaerobic microorganisms according to oxygen accessibility. Simultaneous aerobic and anaerobic degradation of organic wastes in immobilized activated sludge makes it possible to achieve high rates in sewage treatment, stability and adaptability to varying environmental conditions and reduction of excess sludge production as well.

Keywords: immobilized active sludge, methane production and oxidation, wastewater treatment
Microbial diversity in a uranium contaminated environment: the Urgeiriça mine (Central Portugal) as a case study

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An evaluation of the impact of mining activities in the microbial community structure of a former uranium mine was carried out in Urgeiriça (Central Portugal). Samples from the natural environments underground mine and from several stages of the existing water treatment system were studied. The bacterial community structure was based upon the sequencing of the 16s rRNA gene of the isolates. All samples independently of the content in uranium and other metals showed a high number of cultivable bacteria and the isolated population was highly diverse at species level.

Uranium resistant populations were isolated in some of the sampling sites, including the water from the flooded mine and in the lagoon for sludge deposition of this particular contaminated environment, demonstrating a stable uranium resistant population adapted to the mine environment, and the majority of these isolates were affiliated to the Firmicutes phylum. However, in Poço da Cobras where the highest concentration of uranium (8.2 μM) was measured, the vast majority of the isolates belong to the phylum Actinobacteria. When the isolation of strains was performed in R2A medium, without the presence of uranium the majority of the recovered population belonged to the β-Proteobacteria group.

We were not able to isolate strains resistant to chromium (VI), but very different groups of arsenite (III) resistant isolates were recovered.

Microbial populations and CTX-M1 resistance in Escherichia coli isolated from wastewater treatment bioaerosol

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The composition, size, and concentration of the microbial populations comprising the bioaerosol vary with the source, dispersal mechanism in the air, and, most importantly the environmental conditions prevailing at a particular site. In the experiment, samples were collected by means of a sampler MAS-100 Eco. The MAS-100 Eco air monitoring system is a compact sampler intended for use with standard Petri dishes. Petri dishes with respective nutrient media (meat-pepton agar, Endo agar, MacConkey agar with ampicillin and Sabouraud agar) are placed on top of the dish support of the sampler and after aspiration of a preset volume of air, they are incubated at appropriate temperatures. ESBLs were detected by interpretative reading of antibiotic minimal inhibitory concentrations and by PCR. We detected seasonal variations in the concentrations of bacteria. In spring the total count of microorganisms, total coliform and moulds were higher. In workplaces with the highest concentration of microorganisms (coarse treatment, fine mechanical treatment) we detected also antibiotic resistant E.coli with CTX-M1 betalactamase. Resistant microorganisms that exist in bioaerosols in different environments pose hazard to animals and people particularly through exposure to infection antibiotic resistant pathogens or commensals and related potential mortality and failure of therapy.

This study was supported by slovak grant APVV-0028-07.

Keywords CTX-M1, Escherichia coli, bioaerosol
Microbial Screening from Activated Sludge in Degradation of Dimethyl Sulfoxide in Airlift Bioreactor

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DMSO (dimethyl sulfoxide) is a useful and inexpensive environment benign solvent easy to recycle. Its industry-wide adoption has revealed an odorous problem to the industrial parks and their adjacent residential areas due to its decomposition product, DMS (dimethyl sulfide). Our previous research goal is to develop a feasible biological treatment technology to effectively treat the DMSO into oxidative pathway instead of going to the DMS pathway. We have adopted the specific activated sludge as bacterial inoculums to decompose the DMSO into DMSO pathway from the wastewater treatment plant of a DMSO-producing chemical plant. In the performance of airlift bioreactor, the free cell system can degrade the 850-mg L-1 DMSO within 10 hr and has high stability of repeated batch. In this study, we focus on the microbial screening of biological sludge capable of degrading dimethyl sulfoxide (DMSO) in airlift bioreactor were analyzed by using a polymerase chain reaction (PCR-cloning method. Three different suspension conditions from static and dynamic for biological sludge were examined. The bacteria of the different sludge type were found to be Serratia liquefaciens, Brevibacillus brevis, Ochrobacterum sp., Bacillus subtilis, Pseudomonas sp. and Pseudomonas fluorescens which were previously found as denitrifying bacteria, polyphosphate-accumulating bacteria and phenol-utilizing bacteria. From the supernatant of the static sludge or the dynamic sludge, nine strains which could utilize 0.05% (w/w) DMSO with 0.05% (w/w) methanol were isolated and identified. In addition, the newly isolated Pseudomonas sp. might be the most predominant DMSO-degrading microorganism existing in our airlift bioreactor.

Keywords: DMSO (dimethyl sulfoxide); airlift bioreactor; identification; DMSO-utilizing bacteria; PCR-Cloning
Molecular assessment of microbial community structure and dynamics along streams with mixed olive oil and winery wastewaters biotreatment

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The major parcel of biodegradation that occurs during all kind of wastewater biotreatments is performed by microorganisms either from the native microflora or due to added microbial inocula. Although biotreatments are carefully monitored along time in order to assure their efficiency, little attention was paid during decades to the microbial communities present and responsible for the treatments. Besides the need to identify and quantify these microorganisms, it is essential to determine the correlation between their specific metabolic functions and the effectiveness of the biotreatment. The classical approach to characterise a microbial community is based on culturing, identify and count the members of the community and then assign functions to them, according their physiologic characteristics. Nowadays, a large range of molecular methodologies are commonly applied to analyse and compare environmental samples, providing fast and reliable solutions to overcome the bias of culture-dependent methods and allowing a more complete assessment of the microbial community composition and dynamics.

The present work can be considered as a case-study, reporting the biotreatment of mixed olive oil and winery wastewaters. These effluents were chosen, due to their importance in the Portuguese agro-industrial sector, and also to their strong negative impact in the environment.

The experiment was performed in a jet-loop reactor, under aerobic conditions and at room temperature, using native microflora from the crude wastewater as inoculum. Biotreatment was monitored along time, covering the initial start-up batch phase and the continuous regimen, testing two hydraulic retention times (HRT) of 6.0 and 4.5 d. Microbial communities were characterized in samples routinely collected during the biotreatment.

The bacterial community structure was compared using two fingerprinting methods: Temperature Gradient Gel Electrophoresis (TGGE) and Length Heterogeneity-PCR (LH-PCR) analysis of 16S rDNA gene fragments. For TGGE analysis the variable domain V3 of bacterial 16S rRNA was amplified using primers 341F-GC and 538F. For LH-PCR analysis, genomic DNA was amplified with a PCR using a fluorescently labeled forward primer 27F (5'-6FAM) and unlabeled reverse primer 538F. TGGE bands were reamplified and sequenced to identify the community members. Phylogenetic analysis showed the presence of bacteria affiliated with five main phylogenetic groups: alpha-Proteobacteria (40%), beta-Proteobacteria (9%), gamma-Proteobacteria (15%), Firmicutes (20%) and Bacillales (20%). Within these groups, eight genera were identified: Gluconacetobacter, Novosphingobium, Sphingobium, Sphingomonas, Raistonia, Klebsiella, Pseudomonas, Lactobacillus, and Prevotella. Bacterial populations have shown dominance of Gram- groups throughout the entire biotreatment. LH-PCR analysis distinguished nine predominant fragments (468, 471, 474, 496, 499, 521, 524, 555 and 559 bp) in the sample that presented the highest performance (COD removal rates of 67 up to 75%), probably representing the members of the corresponding microbial consortia. Numerical analysis of both TGGE and LH-PCR fingerprinting profiles established five main clusters similar to those higher than 70% (TGGE) or 62% (LH-PCR), showing that the main shifts observed in the microbial community structure were related with changes in tested HRT conditions.

A bioreactor operation depends upon the microbial consortia ability to grow in this man-modified environment whose physical and chemical conditions are subject to numerous and unpredictable fluctuations, that influences the microflora metabolic functions. In this context, TGGE bands corresponding to the samples collected along the biotreatments were correlated with all the environmental data available (TRH, temperature, pH, COD, pO2, Na+, K+, PO43-, NO3−, and NO2−), using canonical correspondence analysis (CCA). Obtained data shows that changes observed on temperature and pH level were the main responsible for the shifts in microbial consortia composition, during the biotreatment.

Furthermore, several raw effluent samples were tested for their metabolic activities using the “Ecoplate” system (Biolog, Inc). Results revealed the presence of microbial communities with marked degradation of phenolic compounds that can be of potential interest for industries applications.

Acknowledgments This work was supported by the FCT Project MOTIVE (FPO/AMB/56616/2004). FCT post-doc grant for S. Chaves (BPD/20819/2004).

Keywords olive oil wastewater; winery effluent; microbial consortia; TGGE, LH-PCR; Biolog

Morphological and ultrastructural peculiarities of ‘Euhalothece natronophila’ cells under different Ci concentrations

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‘Euhalothece natronophila’ is extremely halokalophilic unicellular cyanobacteria, isolated from soda lake Magadi (Samylna A. Ivanovsky, this volume). It obligately requires high concentrations of carbonate salts. Cells of ‘E. natronophila’, incubated in high carbonated medium (high-Ci) containing ≥1 M Na2CO3-NaHCO3, are spherical 2.7-4 μm in diameter. Under conditions of decreased Ci concentration cells transform into very small 3-4 μm spherical cells which have aberrant morphology: thickened irregular long cylindrical forms up to 10-17(23)×3.5-4 μm when 0.2 M CO2-5HCO3-. These cells are pallid and less viable which is indicated also by increase of carotenoid content and decrease of chlorophyll a content in these conditions. According to our data such changes in cell morphology begin under Ci concentration less than 0.8 M (pH 10) even if there is no osmotic pressure changes and total salinity (S) of medium remains high (because of NaCl adding).

We also found that ‘E. natronophila’ morphology depends on pH of medium. Long cylindrical cells occur if increase to pH 11 in high-Ci conditions. As pH change in carbonate solution influences on CO2- and HCO3- correlation, the increase of pH in medium causes decrease of HCO3- according to relation of Henderson-Hasselbalch. Since HCO3- is the only form of Ci available for transport into the cell at high-Ci and pH conditions, it seems that HCO3- limitation is the cause for morphological changes in our experiments.

HCO3- uptake is the result of specific Ci-transporters activity. According to our data there is 3 transport systems (TS) for Ci in ‘E. natronophila’, which differ in their kinetic properties (Samylna A. Ivanovsky, this volume). So it seems to be a good correlation between the formation of inversion cells and the ability of TS in total to provide a cell with necessary amount of Ci under certain conditions.

Ci shortage is a very serious stress factor for extremely halokalophilic and natronophilic cyanobacterium. So its influence on ultrastructure of ‘E. natronophila’ cells has also to be considerable. To investigate it we used cells incubated in low carbonated (low-Ci) medium (0.5 M CO2-5HCO3-, with S = 1.8 M, pH 10-10.5) in comparison with control high-Ci cells (1 M CO2-5HCO3-, S = 1.8 M, pH 10.5-10).

Control cells used as inoculum to both Ci-concentrations are oval. Cell wall layers are clear and there is mucilage perpendicularly to cell surface. Thylakoids are arranged peripherally with a few thylakoids crossing the central part of cell. Inclusions are presented by cyanophycin granules, rare carboxysomes (not over 1-2 per thin section), carbohydrates and other inclusions of different electron density. The interesting peculiarity of ‘E. natronophila’ cells is lamellosomes – polimembrane labyrinth-like structures usually arranged between thylakoids.

Abnormalities in ultrastructure appear already on the 1st day of cultivation under low-Ci conditions. These changes progress later and this enables to mark out several types of cells according to their ultrastructure. Type 1 cells are transparent with friable thylakoids and big quantity of carboxysomes (till 12 per thin section). Cells become larger than control ones and usually have cylindrical morphology with no mucilage around them. Type 2 cells have swollen thylakoids and a lot of lamellosomes. They also have no visible mucilage around cells. Gradually they degrade to the stage when most of the cell contents turn into clots situated near remained thylakoids which surround conglomerates of carboxysomes. Type 3 cells are the most viable as they maintain mostly intact structure. An important peculiarity of low-Ci cells is appearance of additional layer in cell wall. It looks spiral on thin sections and can be solid or in the form of separate scales. All viable (according to ultrastructure) cells in experimental conditions have this additional layer, but if the layer is absent cells degrade.

Morphology of control cells doesn’t change during experiment but their ultrastructure changes in similar way as low-Ci cells. Also 3 types of cells can be distinguished, but type 3 cells prevail over others.

We also see that low-Ci and high-Ci cells may have similar destiny during growth, but abnormalities in low-Ci cells are more crucial and need special adaptations such as additional cell wall layer. Also low-Ci and high-Ci cells change their morphology which is answer to stress conditions and may be related to Ci transport into the cell.

This work is financially supported by RFFI №06-04-00804-a, Presidium of RAS Program (‘Biosphere origin and evolution’) and Contract with Rosnauka № 02.512.12.0027.

Keywords ‘Euhalothece natronophila’, morphology, ultrastructure, Ci cell wall, thylakoids, carboxysomes.
Morphological changes induced by iron in *Chlamydomonas acidophila*.

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Iron is one of the most abundant elements on earth and in the Rio Tinto river, an extreme biotope situated in the southwest region of Spain with a constant very low pH (between 2 and 3), high irradiances, heavy metals and an unexpectedly eukaryotic diversity. Photosynthetic microalgae *Chlamydomonas acidophila*, natural inhabitant of this environment, has adapted to acidic stress by expressing metal tolerance mechanisms. However, little is known about morphological changes induced in *C. acidophila* by exposure to sublethal heavy metal concentrations.

In this work we present an ultrastructural study by electronic microscopy of *C. acidophila* cells to evaluate the effect of iron on cell ultrastructure.

When cells were exposed to Fe$^{3+}$ 0.25, 0.5, 2, 10 and 20 mM, deflagellation was observed and the periplasmalemmal space increased greatly, and also production of mucilage was observed. The most striking modifications of the ultrastructure were seen in the chloroplast and vacuole compartment. *C. acidophila* showed an increased number of small starch grains taking up almost the whole chloroplast; moreover, lipid droplets were also accumulated inside the cells. Thylakoids resulted apparently unaffected by cell incubation in Fe$^{3+}$ added culture medium. The presence of two pyrenoids reduced in size was observed in those cultures treated with Fe$^{3+}$ 0.5 and 2 mM. In those cell cultures incubated with 10 and 20 mM Fe$^{3+}$ iron, the appearance of a new structure consisted of a non-membranous, spherical electron-dense inclusion was observed in vacuoles. Analysis of chloroplast ultrastructure by electron microscopy in cultures incubated with 2 mM Fe$^{3+}$, revealed the presence of lipoprotein particles called “plastoglobules” in the stroma of chloroplasts that appeared as small black globules in close proximity to thylakoids. At the highest Fe$^{3+}$ concentration added to the culture medium (30mM), structures got severely disrupted and normal cell organelles were often hardly distinguishable. All those ultrastructural changes observed in iron exposed cells could be the result of a bioaccumulation process.

Acknowledgements: This work has been supported by the Ministerio de Educación y Cultura (Proyecto AGL2006-12741) and Junta de Andalucía (Proyectos de Excelencia, AGR-4337).

Keywords: extremophiles, *Chlamydomonas acidophila*, ultrastructure, iron

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Natural populations of dominant species and laboratory cultures of cyanobacteria: comparative analysis of amino acid composition and putative significance for primary consumers in aquatic environments

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Cyanobacteria are a very important trophic link in aquatic environments. During the period of mass cyanobacterial bloom they become the major producers in water ecosystems. Amino acids (AA) are an important component in trophic transfer of matter and energy. However, data on amino acid composition (AAC) of dominant species of natural populations of cyanobacteria is very scarce. Researches address to AAC of phytoplankton much less than to that of polyunsaturated fatty acids because the former is commonly considered to be almost constant in contrast to the latter, as shown for the laboratory cultures. In the present study AAC of dominant species of cyanobacteria in a small Siberian reservoir has been investigated and compared with literature data on laboratory cultures.

We found that the dominant species in seston (> 50% of total biomass) were cyanobacteria *Planktothrix agardhii* (Gom.) Anagn., *Gomphosphaeria lacustris* Chod. and *Anabaena flos-aquae* (Lyngb.) Breb. Our study revealed that cyanobacteria are not biochemically homogeneous according to AAC. Total per cent level of essential AA varied from 46.0 ± 1.3% for *Anabaena* to 48.4 ± 0.4% for *Gomphosphaeria*. *Gomphosphaeria* had the highest level of Ala. *Planktothrix* had comparatively low level of Phe, but the highest level of Met. *Anabaena* had no species-specific level of any AA.

Comparing our data with those of laboratory cultures of *Anabaena* (Ahlgren et al., 1992), we found many similarities. Levels of Leu, Val, His, Ala, Gly, Pro, Ile and Thr of the laboratory cultures and the field populations were practically the same. These similarities seemed to support our approach when attributing the biochemical composition of seston samples to dominated cyanobacterial species of the high biomass. Nevertheless, there were some differences in the AAC of the laboratory culture and the field population. Level of Asp in the field population was slightly lower, than those in the laboratory culture. The natural population had significantly higher levels of Lys and Arg, and lower level of Phe, and especially Met and Tyr, than the laboratory culture.

The revealed species specific peculiarities of amino acid composition of the dominant freshwater cyanobacterial taxa may be conditionally limiting factors for the primary consumers.

Keywords: Cyanobacteria, amino acid composition, aquatic ecosystems
Neutonic versus epiphytic bacteria of eutrophic lake and their ability to biodegradation of insecticide deltamethrin

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A layer separating the atmosphere from the water, called the surface microlayer is a specific type of environment, differing clearly from subsurface water both in physical properties and in the chemical and biological composition. Organisms like bacteria, algae and small animals living there are called neuston. The forces of adhesion occurring at the border of the two environments – water and air - contribute to existence of this microlayer. Because of the high concentration of organic substances occurring in the microlayer, both autotrophic and heterotrophic bacteria find optimal conditions for growth. Persistent organic pollutants (POPs) of anthropogenic origin, such as: pesticides, polynuclear aromatic hydrocarbons (PAHs), or polychlorobiphenyls (PCBs) tend to hyperaccumulate in that zone.

Epiphytic bacteria grow on aquatic macrophytes with their greatest numbers occurring in littoral zones of water bodies; this zone is particularly affected by pesticides due to the proximity of farmland. It was demonstrated that only 1-3% of the applied dose of pesticide reaches its target. The majority of the applied compound is wasted and reaches surface waters through runoff, gets dispersed by wind over vast surfaces, or evaporates.

Deltamethrin is a pyrethroid insecticide used widely in agriculture, including vegetable, fruit, and ornamental plant farming, and in forestry to control gnawing and sucking pest. It is a contact and systemic neurotoxin that strongly affects neurotransmitters in the central and peripheral nervous system.

Decomposition of pesticides by microorganisms is an essential process in affecting the fate of pesticides in the environment, finding applications in bioremediation. Microorganisms are highly effective in transforming organic pollutants and modifying their structure and toxic properties; furthermore, they can completely mineralize organic compounds to non-organic products.

This study evaluated biodegradation of the insecticide deltamethrin (1 mg/l) by homogeneous cultures of neutonic (n=25) and epiphytic (n=25) bacteria and by heterogeneous cultures (n=1), which consisted of a mixture of 25 bacterial strains isolated from the surface microlayer (SM=250μm) and epidermis of the Common Reed (Phragmites australis, (Cav.) Trin. ex Steud.) growing in the littoral zone of eutrophic lake Chelmżyńskie (central Poland).

Results indicate that neutonic and epiphytic bacteria are characterized by a similar average capacity to decompose deltamethrin. After a 15-day incubation, bacteria isolated from the surface microlayer reduced the initial concentration of deltamethrin by 60%, while the average effectiveness of the bacteria found on the Common Reed equaled 47%. It was demonstrated that Burkholderia cepacia sp. among neutonic bacteria and Pseudomonas luteola as well as Aeromonas hydrophila among epiphytic bacteria were the most efficient in reducing the concentration of deltamethrin.

Nitrification potential in three different kinds of the Ariake sea sediment and water

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The Ariake Sea located in the west part of Kyushu Island, is a semi-closed shallow sea with macro-tidal and the largest tidal flat in Japan. The sand, silt and mud sediments develop in the different parts of this tidal flat. In this study, we determined nitrification potential rate (NPR) of the sediments and water above the sediments by determining the accumulation of NO₂⁻ and NO₃⁻ in the range of 0-9 cm sediment depth, the NPRs did not correlate to the depth of the sediment sample. NPRs of silty sediment were comparable to that of muddy sediment, but sandy sediment showed much lower NPRs than two other sediments. NPRs of silty, muddy and sandy ranged 7.3-21.1, 12.1-31.7, 0.24-2.9 mmol N/g dry weight sediment (DWS)/h, respectively. NPRs of water also showed the similar pattern to those of sediments. NPR of water above muddy sediment was comparable to that of silty sediment. However, much lower NPR was found in the water above sandy sediment.

The densities of ammonium-oxidizing bacteria in sandy sediment and ammonium concentration in its pore water were lower than those of other two sediments. These results suggested that NPRs in the Ariake sea have correlation to the density of ammonium-oxidizing bacteria and the geochemistry of sediments and water, especially NH₄-N concentration. It is the first report providing the nitrification potential of water and different kinds of sediment from the Ariake sea mud sediment.

Keywords: Nitrification, the Ariake sea, tidal flat, water, sediment, geochemistry.
Nitrogen isotope composition of particulate organic matter (POM) in Lake Kinneret, Israel

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Particulate organic matter collected seasonally throughout the water column of Lake Kinneret exhibited large excursions in POM δ15N in response to the seasonal sequence of dominant N cycle processes in the lake. The nitrification period, during Dec-Jan water column destratification, was characterized by low POM δ15N (as low as -2‰) due to the preference for 14N-NH4 by ammonia oxidizing bacteria leaving water column NH4+ enriched in 15N. Low δ15N POM (and DON) was detected during summer - fall nitrogen fixation period with strong thermal stratification and undetectable DIN in the euphotic zone. The highest δ15N POM values (25‰) were measured during the spring algal bloom and subsequent denitrification period due in part to degradation and mineralization of organic matter but also algal uptake of DIN high in δ15N. δ15N analysis provides a clear 'tag' for the seasonally changing importance of these different N cycle processes. At chemoclines (interfaces), chemosynthetic processes dominate and low δ15N as well as δ13C is localized to this stratum due to strong isotopic discrimination in the uptake of DIC and DIN by chemosynthetic bacteria. δ15N and δ13C measurement provides and ideal tool for locating of the chemocline and identifying the dominant biogeochemical processes associated with it.

Keywords Nitrogen isotopes; Lake Kinneret; POM

Optimization of reaction conditions in binding of magnetic nanoparticles over Flavobacterium ATCC27551

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The unique properties of nanoparticles are allowing their application in modification of microbial cells. Superparamagnetism of magnetic nanoparticles (MNP) is useful for applications requiring manipulation of MNP by a magnetic field. Microbial cells can be magnetically modified by the non-specific attachment of MNP. Alternatively, the modification of cells can be performed by binding paramagnetic cations on acid groups on the cell surface. In many cases the attached magnetic particles or ions do not have a negative effect on viability and phenotype alternation of modified cells. Magnetic modified cells can be immobilized by internal or external magnetic field. Moreover, cells bound on magnetic particles can be stably stored for a long period of time without loss of magnetically property and repeatedly used in process. The microbial degradation of hazardous waste offers a promising strategy by which some wastes may be economically and safely detoxified. Microbial processes can yield precise products, function at low concentrations of solute, and require relatively low levels of technology for construction and maintenance. Organophosphate compounds such as the insecticide parathion (O, O-diethyl-O-4-nitrophenyl phosphorothioate) are degraded by some of bacteria and microbial enzymes. Pseudomonas diminuta MG and Flavobacterium sp. (ATCC 27531) have the ability to degrade a broad spectrum of organophosphorous triesters by virtue of a constitutively expressed organophosphorous hydrolase.

In this study, Flavobacterium ATCC 27551 was used as a model for preparing of magnetic biocatalysts. The magnetic modification of cells was carried out with carboxylate and amino-modified magnetic nanoparticles for covalent coupling by carboxylate and amino-modified magnetic nanoparticles and ionic adsorption by ferrofluids, respectively.

Keywords Flavobacterium ATCC27551 -magnetic nanoparticles- magnetic cell - organophosphates
Pepton hydrolysates of silver carp (*Hypophthalmichthys molitrix*) head as a nitrogen source for *Aeromonas salmonicida* and optimization using Central Composite Design (CCD) and Response Surface Method (RSM)

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Peptone obtained by enzymatic hydrolysis with Protamex of silver carp (*Hypophthalmichthys molitrix*) head waste were used instead of the standard peptones used in commercial Tryptic Soya broth (TSB) media for *Aeromonas salmonicida*. Peptones produced Protamex had a 75% degree of hydrolysis. Optimization of the used peptone as culture media was also done using Central Composite Design (CCD) and Response Surface Method (RSM). Batch experiments were conducted to monitor *Aeromonas salmonicida* growth for duration of 24 hours. A full factorial Central Composite Design of experiments was used to construct second-order response surfaces with the bacterium growth as dependence parameter. The head peptone concentration and the retention time were used as design factors. Adjustment of the quadratic model with the experimental data was satisfactory. Analysis of variance showed a high coefficient of determination value (0.950). It was possible therefore; to develop the empirical equations describing and predicting the optimize value. Results also were shown that enzymatic-modified fish by-products can be used as low cast nitrogen source for bacterial growth.

Keywords: silver carp; peptone; protamex; Response surface method; central composite design

Photocatalysis / biotreatment coupling for the removal of biorecalcitrant compounds

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With the development of intensive agriculture and industrial activities, an increasing amount of chemical compounds including pesticides (insecticides, fungicides, weed killers), antibiotics... is employed. As a consequence, more and more toxic and non biodegradable compounds accumulate in natural waters and their concentrations levels could reach beyond the standards fixed by the European authorities. Hence, it became a necessity today to treat these polluted waters. For this purpose, many techniques exist, including physical treatments (such as adsorption on activated carbon) or chemical ones (oxidation processes). Such processes just transfer the pollution or are relatively expensive. More efficient and cost-effective processes have been considered, such as the coupling of Advanced Oxidation Process (AOP) and biological treatment (Scott and Ollis, 1995), which is justified by the need to reach a total mineralization of pollutants at lower costs with short processing times. For concentrated effluents (unused treatment solutions, spray, machine and pesticide container washing, industrial wastewater), integrated process involving photocatalysis and biological treatment was considered in this study. It was applied to the elimination of antibiotics (Tetracycline and Tylosine), pesticides (Cyproconazole, Isoproturon, Dimethoate), synthetic hormones (Ethynylestradiol) and even plasticizers (Bisphenol-A).

The effluent containing the target organic compound is firstly pretreated by photocatalysis in order to decrease its toxicity and increase its biodegradability. The irradiated effluent is then treated by biological way in order to ensure the mineralization of the remaining products after the photocatalysis step. One of the key parameters that should be optimized is the irradiation time during the photocatalysis step. Indeed, the objective is to reduce the concentration of the target molecule until reaching negligible toxicity towards bacteria, while increasing as high as possible the biodegradability of the irradiated solution, which can be monitored through the measurement of the BOD. To ensure sufficient organic material for the subsequent biological treatment, a low mineralization rate should be achieved, which can be monitored through the measurement of the total organic carbon (TOC). The Chemical Oxygen Demand (COD) on TOC ratio (COD/TOC) evolution allowed following the oxidation state of the considered molecules. The irradiated effluent obtained is subsequently biologically treated.

Keywords: Biorecalcitrant pollutants; integrated process; Photocatalysis; Biodegradability; Toxicity.

References

Procaryotic biodiversity in anaerobic digester treating municipal solid waste
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Millions of tons of municipal solid waste (MSW) are daily produced worldwide. Two of the greatest challenges of our society are to reduce the organic pollutants released and to increase the energy production from renewable sources. Biomethanization (anaerobic digestion) of the organic fraction (OF) of the MSW becomes a good solution to resolve both problems. Anaerobic digestion comprising various stages: hydrolysis, fermentation, acetogenesis and methanogenesis, and it is the consequence of the combined and coordinated metabolic activity of bacteria and archaea. The use of molecular tools to biomonitoring digesters is an excellent approach to know the microorganisms present during the process which later can be useful improving the efficiency of organic matter degradation, methane production and stability of the process. To extending our understanding on the biomethanization process, the bacterial and archaeal biodiversity of an anaerobic digester treating OF-MSW was identified and quantified by rRNA-based complementary methods: Fluorescence in situ hybridization (FISH), cloning, amplified ribosomal DNA restriction analysis (ARDRA) and sequencing of 16S rDNA. The sample was collected from a Continuously Stirring Tank Reactor (CSTR) treating the OF-MSW operating for one year at 35°C (mesophilic), and pH around 6.5 to 7.5 at Madrid (Spain). For FISH, hybridization was performed using the universal probes EB338 and AR915 for bacteria and archaea respectively, and NON138 as negative control. The total cells present in the samples were determined by direct counting DAPI stained cells. For clone libraries construction, total DNA was extracted from approximately 1g of digested OF-MSW and used to bacterial and archaeal 16S rDNA amplification by PCR. Amplicons were purified and cloned into the PGEM-T vector. Plasmids containing 16S rDNA were extracted and screened by ARDRA using the endonuclease BfoC1. Clones with the same restriction pattern were grouping together and one representative was sequenced and analyzed. The number of microorganisms reached 4,3x109 cells (total DAPI stained cells/g OF-MSW digested). Of those, 80-85% corresponded to the domain Bacteria and 15-20% to Archaea. Several morphologies: rods, long bowed rods and cocci were visualized. The bacterial and archaeal libraries were obtained with successful. ARDRA screening showed 19 and 20 different bacterial and archaeal patterns, respectively. Although the ARDRA analysis shows an apparent high biodiversity, this can be only accurately confirmed by sequence analyses of the 16S rDNA sequences. The amplicons are being presently sequenced and the results will be presented in the Congress.

Keywords:Anaerobic digester . Municipal solid waste . 16S rDNA . Clone library . ARDRA . FISH.

Production of Prodigiosin for Serratia marcescens in Residues Agro industrials
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Prodigiosin is an antibiotic, characterized by the biosynthesis of the red pigment known by tripyrrole, produced by several bacteria’s mainly Serratia marcescens. This pigment is a due promising drugs your characteristics antifungal, antimicrobial, antitumor and immunosuppressive. The present work had as objective the production and identification of the prodigiosin for S. marcescens in medium agro industrials. The sample was isolated of the soil of plantation of banana trees in the state of Pernambuco and deposited in the NPCIAMB/UNICAP-PE. The bacterium was sowed in Petri plates in medium of residues agro industrials: cassava flower (6%) and corn step (6%) added of 2% of mannitol, at 24-48 hours to 28°C. The pigment was isolated and purified by thin-layer chromatography (TLC), with plates of silica gel, and soon after for a column chromatography (50.0 x 1.0 cm). The production of the pigment was of 357mg/l and 495mg/l using cassava flower and corn step residues respectively and using broth medium the production in Erlenmeyer flasks of 250ml with 100ml of variables 23 - 27 µg/ml in the static for cassava flower and corn step. For the confirmation of the pigment prodigiosin the following analyses were used: spectrophotometer UV and antimicrobial for biological chromatography using plates inoculated with Bacillus subtilis. In the present study, the production of the prodigiosin were satisfactory, when was submitted to the medium with agro industrials residues economically viable in terms of the yield prodigiosin, the extracted pigment presented activity antimicrobial.

Keywords: prodigiosin, Serratia marcescens, cassava flower, corn step

Supported by CNPq, CAPES and FACEPE.
Prokaryote-Virus Coexistence Model in the Deep Ocean

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In the deep (>3000 m depth) ocean, there are approximately 10^3-10^5 cells of prokaryotes and 10^5-10^6 particles of viruses per mL, and almost all biomass consists of heterotrophic prokaryotes. More than 95% of the organic matter are smaller than 700 nm in diameter, and comprise various non-living particles, prokaryotes and viruses. These organic matter, prokaryotes and viruses can be treated as particles. The carbon concentration of the organic matter in the deep ocean is about 40 μM [1], which is extremely low to sustain active growth of prokaryotes, and the estimated age of the organic matter based on 14C is 4000-6000 y [1]. More than 80% of the decay of prokaryotes in the deep ocean are caused by the viral lysis [2]. How quickly do prokaryotes grow, and how quickly are prokaryotes lysed by viruses in the deep ocean? How can prokaryotes and viruses coexist in the deep ocean? As indispensable factors for the propagation of prokaryotes and viruses, the interaction between prokaryotes and organic matter and/or viral particles are important. The interactions would mainly be collisions between each particle. We hypothesized that these interactions depend on the collision frequency between prokaryotes and organic matter and/or viruses. At a simple calculation based on solely Brownian motion, the vertical movement of prokaryotic cells might be negligible. The purpose of this work was to answer these questions by constructing a model.

To estimate the sedimentation velocity of prokaryotic cells, we measured the buoyant density of the cell instead of measuring the mass, since the mass of one cell is too small to measure directly. The buoyant density of the prokaryotic cell was 1.047-1.067 g cm^-3. From this value, we estimated that the cell sinks at a rate of 10-100 μm h^-1 in the seawater (1.027 g cm^-3) [3]. This sedimentation movement increased the collision frequency between prokaryotes and organic matter (500 μm) to 1.4-folds, however, it did not affect the frequency between prokaryotes and viruses [3]. In the deep ocean, the doubling time of prokaryotes was estimated as 6.0-10 y under the assumptions of the sedimentation (100-10 μm h^-1), carbon mass of the cell (10 fg) [4], and the cell number (10^6 cells mL^-1). This value is comparable to the estimation (an order of 1 y) by using the average age of organic matter (4800 y) [1].

Then, we constructed a model with various growth rates of prokaryotes. In the model, three types of prokaryotes (A, B, and C) and three types of virus (X, Y, and Z), whose host is A, B, and C, respectively, are considered. Prokaryote A and B are heterotrophic and C is autotrophic. The doubling time of A, B, and C are assumed to be 1.0 y, 1.5 y, and 120 d, respectively. Prokaryote A assimilates Y and Z, and B assimilates X and Z, when heterotrophic prokaryotes collide with viruses, A, B, and C are spontaneously infected by X, Y, and Z respectively when viruses collide with their host prokaryotes. All the prokaryotes are supposed to burst at 1.5 doubling time of the recipient cell after infection. The number of prokaryotes and viruses, and the Shannon’s diversity index of 300 years were examined in this model. As a result, all prokaryotes are able to coexist with viruses through the examined period (prokaryotes: 0.3-1.5×10^6 cells mL^-1; viruses: 0.1-6.0×10^6 particles mL^-1), and at the same time, the Shannon’s diversity index of both prokaryotes and viruses were also maintained (prokaryotes: 1.0-1.6; viruses: 0.5-1.6).

In conclusion, this is the first report of the prokaryote-virus coexistence model in the deep ocean by treating prokaryotes, viruses, and organic matter as particles and by taking into consideration the sedimentation velocity of prokaryotes.

References


Keywords: prokaryotes; viruses; increase and decrease; diversity; deep ocean.

Protozoan community dynamics in an intermittent feeding and aeration bioreactor

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The effects of operation conditions of an intermittent feeding (at the anoxic phase) and aeration bioreactor (fed with wastewater from the municipal plant of the city of Xanthi, North Greece) on the protozoan community dynamics and structure were investigated. A 45-L glass bioreactor operating under an intermittent aeration of 25 and 35 min aerobic treatment, Vas. Sofias 12, 67100 Xanthi, Greece

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The effects of operation conditions of an intermittent feeding (at the anoxic phase) and aeration bioreactor (fed with wastewater from the municipal plant of the city of Xanthi, North Greece) on the protozoan community dynamics and structure were investigated. A 45-L glass bioreactor operating under an intermittent aeration of 25 and 35 min aerobic treatment, Vas. Sofias 12, 67100 Xanthi, Greece

In the deep (≥3000 m depth) ocean, there are approximately 10^3-10^5 cells of prokaryotes and 10^5-10^6 particles of viruses per mL, and almost all biomass consists of heterotrophic prokaryotes. More than 95% of the organic matter are smaller than 700 nm in diameter, and comprise various non-living particles, prokaryotes and viruses. These organic matter, prokaryotes and viruses can be treated as particles. The carbon concentration of the organic matter in the deep ocean is about 40 μM [1], which is extremely low to sustain active growth of prokaryotes, and the estimated age of the organic matter based on 14C is 4000-6000 y [1]. More than 80% of the decay of prokaryotes in the deep ocean are caused by the viral lysis [2]. How quickly do prokaryotes grow, and how quickly are prokaryotes lysed by viruses in the deep ocean? How can prokaryotes and viruses coexist in the deep ocean? As indispensable factors for the propagation of prokaryotes and viruses, the interaction between prokaryotes and organic matter and/or viral particles are important. The interactions would mainly be collisions between each particle. We hypothesized that these interactions depend on the collision frequency between prokaryotes and organic matter and/or viruses. At a simple calculation based on solely Brownian motion, the vertical movement of prokaryotic cells might be negligible. The purpose of this work was to answer these questions by constructing a model.

To estimate the sedimentation velocity of prokaryotic cells, we measured the buoyant density of the cell instead of measuring the mass, since the mass of one cell is too small to measure directly. The buoyant density of the prokaryotic cell was 1.047-1.067 g cm^-3. From this value, we estimated that the cell sinks at a rate of 10-100 μm h^-1 in the seawater (1.027 g cm^-3) [3]. This sedimentation movement increased the collision frequency between prokaryotes and organic matter (500 μm) to 1.4-folds, however, it did not affect the frequency between prokaryotes and viruses [3]. In the deep ocean, the doubling time of prokaryotes was estimated as 6.0-10 y under the assumptions of the sedimentation (100-10 μm h^-1), carbon mass of the cell (10 fg) [4], and the cell number (10^6 cells mL^-1). This value is comparable to the estimation (an order of 1 y) by using the average age of organic matter (4800 y) [1].

Then, we constructed a model with various growth rates of prokaryotes. In the model, three types of prokaryotes (A, B, and C) and three types of virus (X, Y, and Z), whose host is A, B, and C, respectively, are considered. Prokaryote A and B are heterotrophic and C is autotrophic. The doubling time of A, B, and C are assumed to be 1.0 y, 1.5 y, and 120 d, respectively. Prokaryote A assimilates Y and Z, and B assimilates X and Z, when heterotrophic prokaryotes collide with viruses, A, B, and C are spontaneously infected by X, Y, and Z respectively when viruses collide with their host prokaryotes. All the prokaryotes are supposed to burst at 1.5 doubling time of the recipient cell after infection. The number of prokaryotes and viruses, and the Shannon’s diversity index of 300 years were examined in this model. As a result, all prokaryotes are able to coexist with viruses through the examined period (prokaryotes: 0.3-1.5×10^6 cells mL^-1; viruses: 0.1-6.0×10^6 particles mL^-1), and at the same time, the Shannon’s diversity index of both prokaryotes and viruses were also maintained (prokaryotes: 1.0-1.6; viruses: 0.5-1.6).

In conclusion, this is the first report of the prokaryote-virus coexistence model in the deep ocean by treating prokaryotes, viruses, and organic matter as particles and by taking into consideration the sedimentation velocity of prokaryotes.
Pseudomonas arsenicoxidans sp nov., arsenite-oxidizing strain, isolated Atacama desert

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A Gram-negative, aerobic bacterial strain, designated VC-1, was isolated from sediments samples from Camarones valley, Atacama Desert. This sector presents high arsenic concentration, both water (1100ug/L) and sediments (550 ug/L). This strain was able to tolerate 5 mM As(III) and was able to oxidize the 100% of arsenite present in the medium, 1,77 mg ml-1 after 48 hours of incubation, using lactate as carbon source (Figure 1). Oxidation assays, were evaluated by HPLC/HG/QAAS. In addition aox genes, genes essential for arsenite oxidation, were detected by PCR. In order to check first for the identity of the new isolate, we studied the phylogenetic affiliation based on 16S rRNA gene sequence comparisons. For this, bacterial DNA isolation, PCR amplification using the pair of primers GM3 and GM4 and sequencing of the 16S rRNA gene were carried out as previously described. For further taxonomic studies we used the reference strain isolated from the Swedish collection of microorganisms CCUG. In order to clarify the genealogy of the new isolate, we performed a MLSA by using four additional housekeeping gene sequences (ITS, ggyB, rpoD, fusA, recA) were obtained for all the studied strains. Tree topologies were compared and validated by the use of the different algorithms. Bootstrap values were performed by using the program PHYML with a total of 100 replicates. The resulting tree shown in figure 2, indicates that the strain VC-1 forms an isolated branch within the set of strains studied, and results in accordance with the previous 16S rRNA gene sequence tree reconstructions. In order to prove that the strain VC-1 was representing a new species of Pseudomonas, we performed DNA-DNA hybridization experiments (DDH) as previously described. The levels of DDH similarity between the new isolate and the type strains selected were shown in table 1. In all cases, the results were below 63.5%, as an indication that the strain could be classified as a new taxon. In addition, we studied the fatty acid profiles of VC-1. All strains were simultaneously grown at 28ºC for 2 days on Tripticase Soy agar (TSA) prior to analysis. The different patterns observed did not differ very much among each other. The most remarkable features in the profiles were the common high abundances of the fatty acid C16:0. The complete set of taxonomic determinations indicated that VC-1 formed an independent line within the Pseudomonas sensu stricto, and was different enough from its closest relatives by means of both genetic and phenotypic characters to be classified as a new species of the genus. We therefore propose the strain VC-1 to be the type strain of the new species Pseudomonas arsenicoxidans sp nov. Finally, the significative oxidation capacity shown by VC-1 strain opens the way to further studies aimed at implementing biological systems to treat arsenic rich wastewater.

Keywords: arsenite-oxidizing, Pseudomonas arsenicoxidans

Figure 1. Growth in a chemically defined medium containing arsenite (500 µg/ml) of VC-1 strain. Arithmetic plot ▲ absorbance; ◆ arsenite concentration.

Figure 2. tree reconstruction based on the concatenated alignment of all six genes included in the MLSA approach and listed in table X. The alignment of 4813 homologous positions was used to calculate the tree by using the maximum likelihood algorithm as implemented in the ARB program package. The tree topology was identical to that reconstructed with PHYML and RAxML. Bootstrap values were calculated after 100 tree reconstructions.

Quantification and microbial toxicity testing of pharmaceuticals in tropical marine sediments, All Saints Bay, Bahia, Brazil

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Pharmaceuticals in the environment have gained increased attention during the last decade. The main routes by which these compounds enter aquatic ecosystems are municipal wastewaters, but they are also introduced to the environment by disposal of unused or expired medicines, wastewater from manufacturers and landfill leachates. As pharmaceuticals are developed to have some kind of biological function and persistence in the organisms applied to, they also have a potential to bioaccumulate and induce effects in aquatic and terrestrial ecosystems. Monitoring studies have shown that pharmaceuticals and their metabolites are very resistant to most water treatment techniques and are present in all kinds of aquatic systems.

Biodegradation by microorganisms is the main mechanism to eliminate organic compounds from the environment. The main groups involved in degradation processes are bacteria and fungi, the latter being predominant in soils, while bacteria are of major importance in the aquatic environment, including wastewater treatment. Bioactive compounds as pharmaceuticals may have toxic effects on bacteria, especially the antibiotics that are designed to combat bacterial diseases and thereby may interfere with the degradation processes of these substances in the environment. Data on toxic effects of pharmaceuticals on sediment bacteria are necessary to evaluate the fate, persistence and thereby the risk inherent to these compounds in the environment.

In Brazil, data on the presence of pharmaceuticals in the aquatic environment are still scarce, while the consumption of these compounds suffers little control and disposal of expired drugs is indiscriminate. The Brazilian government does not make demands on security or quantity limits regarding the discharge of pharmaceuticals to water bodies. Most of the data available refer to the subtropical South of Brazil.

Pharmaceuticals present in tropical marine sediments were quantified in the All Saints Bay (BTS), Salvador, state of Bahia, for the first time during this year. Seventeen sampling points were chosen and the following compounds were detected (in order of frequency of appearance): Atenolol (100%), Ibuprofen (100%), Diclofenac (94%), Diazepam (64,7%), Carbamazepine (41%), Erythromycin (29,4%). The concentrations ranged from less than 0,10 to 18,84 ngg-1 (dry weight).

Parallel to the quantification of pharmaceuticals in the sediments, we have started to screen the toxic effects of the identified compounds to sediment bacteria, using plate count techniques, and the data on microbial toxicity testing will be presented at the International Conference on Environmental, Industrial and Applied Microbiology (BioMicroWorld2009).

Keywords pharmaceuticals; tropical marine sediment; microbial toxicity
Red pigments producing novel marine bacterial species *Zooshikella rubidus* S1-1

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A marine Gram-negative, red-pigment producing bacterial strain, designated S1-1 T, was isolated from tidal flat sediment of the Yellow Sea, Korea. On the basis of phenotypic properties, phylogenetic distinctiveness and genetic data, strain S1-1 T was classified as a novel *Zooshikella* species, for which the name *Zooshikella rubidus* sp. nov. was proposed.

The red-pigments of the strain S1-1 T were identified as prodigiosin, cycloprodigiosin, and two novel compounds containing seven prodigiosin analogues by LC-MS/MS analysis. The strain S1-1 T was produced both prodigiosin and cycloprodigiosin as a major metabolite. These compounds have broad spectrum antimicrobial activity and anticancer activity against human melanoma cell. Especially, cycloprodigiosin show about two times higher antimicrobial activity than prodigiosin.

**Keywords** marine bacteria; *Zooshikella rubidus*; prodigiosin; cycloprodigiosin; LC-MS/MS; antimicrobial; anticancer

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Relationships between hydrophobicity and biofilm formation in *Streptococcus agalactiae* strains

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**Background:** *Streptococcus agalactiae* GBS, is a human pathogen, that as recently demonstrated from us forms biofilms in vitro with variable efficiency [Roccasalva L.S., et Al. Giorn. It. Ost. Gin. 30: 301-306, 2008]. Aim of this study was to investigate the relationships between hydrophobicity and the ability of GBS to form biofilm.

**Methods:** 20 strains of GBS were investigated for the purpose. All the strains were identified by means of biochemical and molecular tests. Biofilm experiments were performed in 96-well polystyrene microtiter containing broth medium. Experiments were performed in unmodified atmosphere and Todd-Hewitt Broth (THB). Increasing concentrations of glucose (1 g/L, 25 g/L, 50 g/L and 75 g/L) were also investigated for their influence on both hydrophobicity and biofilm production. The quantitative measurements was achieved by means of colorimetric methods using crystal violet. The optical density of the biofilm was measured at 570 nm (OD$_{570}$) by the calculation of biofilm index (BI), the cut-off was established at OD 0.061. The strains were then divided by means of BI into four categories: no producers (BI < 0.061), low producers (BI > 0.061 and < 0.120), moderate producers (BI >0.121 and < 0.300, and heavy producers (BI > 0.300). The hydrophobicity was investigated by using the hexadecane test, and expressed as percent of hydrophobicity.

**Results:** By varying the concentration of glucose in the culture medium, the hydrophobicity varied little in most cases, nevertheless the BI increased with increasing concentration of glucose. Most of the strains had a hydrophobicity index of > 80%. We have not find any correlation between hydrophobicity and biofilm formation (for the same hydrophobicity, there are strains with very different BI), in our experimental conditions.

**Conclusions:** We can conclude that biofilm formation in GBS is not correlated with hidrophobicity.

**Keywords:** *Streptococcus agalactiae*, hydrophobicity, biofilm
Response of *Pseudomonas* to low iron concentrations in presence of sodium benzoate

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Iron is an essential element for most microorganisms, owing to its importance in a variety of biochemical reactions, including respiration, photosynthetic transport, nitrate reduction, chlorophyll synthesis, nitrogen fixation and detoxification of oxygen radicals. Despite being the fourth most abundant element in the earth’s crust, iron is frequently a growth limiting nutrient. In an aerobic environment and at physiological pH, iron is present in the ferric state and forms insoluble hydroxide and oxyhydroxide precipitates. Bacteria have evolved several different mechanisms to acquire this essential nutrient. The most common acquisition mechanism is the use of low molecular weight, high affinity chelators termed siderophores.

Coastal sand dunes are stressed and extreme environments in terms of nutrient availability. Siderophore producing isolate TMR2.13 identified as *Pseudomonas* was isolated from the rhizosphere zone of *Ipomea pes-caprae* found along the coastal areas of Goa - India. The isolate was found to be a strong siderophore producer as revealed by the Chrome azurol sulphonate (CAS) plate assay. Furthermore, its pigment production was studied in a nutrient rich and a defined Mineral salts medium (MSM) supplemented with glucose or benzoate as the sole carbon source and in presence and absence of iron. The isolate was found to produce varied pigment patterns when grown in above mentioned media. The pigment production was studied by UV-Visible spectrophotometry of the culture supernatants. Effect of various iron concentrations on pigment production revealed that the isolate did not produce pigment when FeSO₄ was supplemented at a concentration above 15mg/lt. Interestingly, the pigment produced in MSM in absence of added iron showed absorption maxima at 408nm. The effect of media and carbon sources on the production of pigment, on probable siderophore and the effect of iron on growth and pigment production in presence of benzoate will be presented.

**Keywords** siderophore; sodium benzoate; *Pseudomonas*; chrome azurol sulphonate

Responses of aquatic microbial decomposers to inorganic nutrients in a warming scenario

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The Intergovernmental Panel on Climate Change (IPCC, 2007) projected that the global temperature is expected to rise between 1.1 °C and 6.4 °C till the end of this century. The climate models indicate that the temperature changes in Northwest Europe will entail increased drought periods followed by intense rainfalls. Therefore, nutrient inputs to freshwater ecosystems from the surrounding terrestrial soils are expected to increase, enhancing eutrophication. The aim of this study was to examine the interactive effects of temperature and inorganic nutrients on organic matter turnover and activity of the associated microbial decomposers in streams. Freshly fallen leaves were immersed in a reference stream in the Northwest Portugal, during 10 days, to allow microbial colonization and then were exposed in microcosms to 6 levels of nitrate (0.09-5 N-NO₃ mgL⁻¹) and 3 levels of phosphate (0.003-0.3 P-PO₄ mgL⁻¹), alone or in all possible combinations. One set of microcosms was kept for 19 days at a temperature typically found in the stream during autumn (12 °C) and the other at 18 °C to simulate a warming scenario. The increase in temperature stimulated microbial activity on decomposing leaves, assessed as fungal biomass and sporulation, and enhanced leaf mass loss. At both temperatures, microbial decomposition of leaf litter was augmented by increased nitrate concentrations, but not by phosphate. Increased nutrient concentrations stimulated fungal biomass and sporulation and a hump-shaped relationship between nitrate concentration and fungal biomass or sporulation was found at the highest temperature. This suggests that high nutrient concentrations may limit fungal growth and reproduction under the predicted warming scenario. However, moderate nutrient inputs in streams might enhance microbial activity leading to faster leaf decomposition and nutrient turnover.

**Keywords** leaf decomposition, microbial decomposers, inorganic nutrients, warming, streams

**Acknowledgements.** The Portuguese Foundation for Science and Technology supported this work (PTDC/CLI/67180/2006) and I. Fernandes (SFRH/BPD/42215/2007).
Robust microbial community for treatment of ammonium-rich wastewater

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Water and sediment samples were collected along the flow path of a pilot-scale ammonia purification system (Fig. 1), which was designed for biological removal of 5 mg per liter ammonium from wastewater. Samples, including background samples, were collected 3 times: during equilibration of the system or 2 months before the operation started (March 2007); and 16 months after the operation started (August 2007 and September 2008, respectively). Samples were collected into pre-steriled 2 L Pyrex bottles and transferred to the Oak Ridge National Laboratory, Oak Ridge, TN on ice and were processed immediately. The total community genomic DNA was extracted from water follow the method described efzer (1) or from sediment using the PowerSoil™ DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA). The microbial communities from 2 time points (March and August, 2007) were analyzed using cloning and Sanger sequencing. However the 454 FLX pyrosequencing method was used to study the microbial communities from September 2008 samples. A total of 36 samples were studied using either Bacteria or Archaea primers. All collected samples were also analyzed for the presence of ammonium using Low Range Ammonia test tubes (HACH Company, Loveland, CO). The results of this study showed that after sixteen months up to 25% of the 5 mg per liter ammonium was oxidized in the trickling filters (TF), 60% in splitter box (SB) and extraction trenches (ET) that contained zero-valent iron with the remaining 15% of the ammonium oxidized in settling pond and wet lands (Fig. 1). The removal of ammonium in the system was efficient and essentially complete. Ammonia at the exit gate was 0.27 mg per liter after three months (August 2007) and bellow detection limits after equilibration (September 2008). The microbial community analysis revealed the significantly elevated amount of ammonia-oxidizing bacteria and archaea as well as nitrite-oxidizing and denitrifying bacteria in the TF, SB, and ET samples. Among bacteria Nitrosomonas, Nitrosospira (NH₄⁺ => NO₂⁻), Nitrobacter (NO₂⁻ => NO₃⁻), and Thiobacillus (NO₂⁻ => NO₃⁻) were identified. Ammonia-oxidizing archaea were from the phylum Crenarchaeota. In the wetlands, which represent compost ponds, the Planctomycetes (anaerobic NH₄⁺ oxidation) and Helicobacteraceae (use NH₄⁺ and NO₃⁻ as the nitrogen source) were identified. These bacteria and archaea were not detected in background samples and their populations decreased to detection limits in the gate samples. Tracking the process, showed that changes in microbial communities were initiated by ammonium injection and directed to the development of a microbial nitrification / denitrification cycle.

Fig. 1. Schematic diagram of the pilot treatment system. Water flow is indicated by arrows. Dots show the sampling sites.


Keywords: Wastewater, Ammonium, Ammonia-oxidizing Bacteria and Archaea

Role of Catalases in Isolates of Comamonas from a Polluted Environment

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Bacteria of the family Comamonadaceae are known for their broad catabolic diversity in the degradation of various xenobiotics and are very often present in polluted environments. Many contaminants in the soils are subjected to common environmental oxidation processes, resulting in the formation of reactive intermediates, mainly radicals that react with oxygen to produce the reactive oxygen species (ROS). For the survival of individual isolates of the gram-negative bacteria C. testosteroni CCM 1931, C. terrigena K3, C. terrigena N3H, C. terrigena NH₃ and C. terrigena CCM 2409, the production of catalase and diaminoperoxidase activity was found to be important. We have evaluated the role of monofunctional and bifunctional heme-containing catalases from the soil bacteria in the response to various forms and levels of oxidative stress. Regulation of catalase and peroxidase activity, including changes in the expression of isozymes, proves to be important for the organism in surviving such challenges. Sequence analysis confirms the presence of highly conserved catalase sequence motifs in two environmental strains of C. terrigena but no such sequence motif could be detected in those strains that were not exposed to oxidative stress. The differences in the primary structure of catalases may influence catalase expression in the oxidative stress defence mechanism in bacterial cells.

This study was supported by the slovak grants APVV-0444/07 and VEGA 2/0084/08.

Keywords: polluted soils, bacteria; catalases
Role of Photochemically-Induced Oxidative Stress in Determining the Biological Effects of UV Radiation in Bacteria

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Continuing decreases in stratospheric ozone concentrations have led to extensive concern about its ecological consequences, mostly related to the coconcomitant increase in UV radiation reaching the Earth’s surface. Due to their small size, the fact that their genome comprises a large portion of their cellular volume and that they have short generation times, bacteria are particularly susceptible to UV-induced damage. Since they play a crucial role in the cycling of organic matter and energy in aquatic ecosystems, depicted in the concept of the microbial loop, the study of the effects of UV radiation (UVR) on bacteria and its consequences to the aquatic trophic webs becomes of major importance.

The effects of UVR on bacteria can be direct, as a consequence of UV-direct damage to biomolecules, or indirect as a result of the cellular oxidative damage exerted by free radicals generated from UV-induced photochemical transformation of organic matter. However, the information on the contribution of the indirect pathway to UV-induced bacterial inactivation is virtually unknown.

At the surface microlayer (SML), i.e., the top millimeter of the water column thrives a bacterial community, generally termed bacterioenaston, exposed to high intensities of solar UV radiation. Furthermore, accumulation of pollutants and dissolved organic matter (DOM) exposed to high solar UV radiation results in the production of photo-oxidants that might impair bacterial metabolism. Nonetheless, enhanced bacterial abundance and activities in surface waters suggest that bacteriocenston might have adapted to both UV-induced direct damage and oxidative stress, making the SML an interesting environment for the assessment of UV effects on prokaroytes.

The objective of this work was the evaluation of the role of UV-induced oxidative stress resulting from the photochemical transformation of organic matter, in determining the effects of UV radiation on bacterial abundance and activity. For that, bacteria-free organic matter suspensions (0.02μm filtered water) from the SML and underlying water (UW) of the estuarine system Ria de Aveiro (Portugal) were exposed to natural solar radiation (cumulative exposure: 9.2 KJ m⁻²) and inoculated (1:10 dilution) with 9 different bacterial isolates isolated from the same ecosystem. Cell suspensions were incubated in the dark and cultivable bacteria, bacterial heterotrophic activity (determined by the H-Leuincration method) and intracellular ROS generation (detected using the probe DCFH-DA) was monitored after incubation and compared to the values obtained before inoculation and in non-irradiated (dark) controls.

Incubation of bacterial isolates in irradiated organic matter suspensions resulted in increased colony forming units (CFUs) and heterotrophic activity probably as a result of enhanced bioavailability of low molecular weight compounds (LMWC) in irradiated samples. In general, the increase in colony counts and the stimulation of heterotrophic activity was higher in isolates inoculated in irradiated SML water suggesting a nutrient environment distinct from subsurface waters. Oxidative stress indicators revealed an increase in intracellular ROS generation (up to 30%) and lipid peroxidation (as high as 60%) during the time course of the dark incubation demonstrating that irradiation of DOM also results in the formation of prooxidants that negatively impact bacterial biochemistry, and ultimately could make bacteria more susceptible to other types of environmental stress. Furthermore, detection of higher levels of oxidative stress indicators in all the isolates inoculated in irradiated UW (comparatively to SML) suggest that irradiation generates a “favorable” LMWC-rich environment in irradiated SML water and a more “stressful” free radical-rich environment in irradiated underlying waters.

Contrarily to current knowledge, these results might indicate that underlying waters are more likely to induce photo-oxidative stress on bacteria than surface waters, where intense levels of naturally occurring UVR could lead to the decomposition of organic matter and pollutants into simpler forms, less toxic to bacterial cells that might actually promote bacterial growth.

Sacharomyces cerevisiae UE-ME3 is a good strain for isoproturon bioremediation?

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Isoproturon is an active component of several pesticides applied in Autumn-Winter crops which persist occasionally in the soil, aquifers and biological systems, at levels higher than established by directives of European Community. Biotransformation of several organic compounds like this toxic phenylurea greatly contributes to the ROS formation and resulting cell death in aerobic organisms. The cell enters into chronic oxidative stress when the ROS production exceeds the endogenous antioxidative defences involving tripeptide glutathione (GSH), glutathione reductase (GR), glutathione peroxidase (GPx) and glucose-6-phosphate dehydrogenase (G6PD) activities. In general, changes in the value of the GSH/GSSG ratio, GR, GPx and G6PD activities are early indicator of sensitivity to oxidative stress. In other hand MDA level is also used as marker of lipid peroxidation in different biological systems. Consequently, the work here described aimed to evaluate the physiological and antioxidative response of wild-type Sacharomyces cerevisiae (UE-ME).

Yeast cells growing to mid-exponential phase at 28 °C in YEPD medium with 2 % (w/v) of glucose were inoculated in the same conditions and allowed to grow in the absence or presence of 5, 25, 50 and 100 μM isoproturon, during 72 h. The cultures were used to obtain the post-12000 g supernatant, which was used for determination of GSH, GSSG and MDA contents as well as enzymatic activities were compared by ANOVA one-way, followed by Duncan test to identify significant differences (p<0.01).

The experimental results shows a bimodal adaptive response of S. cerevisiae UE-ME, given that yeast grown in the presence of 50 and 100 μM of phenylurea display in stationary-phase growth rate higher than yeast control or exposed to 5 and 25 μM isoproturon. Furthermore S. cerevisiae UE-ME, grown in the presence of isoproturon 5 μM, show a decrease of cytoplasmic GSH/GSSG ratio, an increase of cytoplasmic MDA level as well as an increase of GR and GPx activities. These facts point us that yeast cells exposed to 5 μM isoproturon are coming in oxidative stress occurring increased of cell damages and cell death, possibly by an active process.

S. cerevisiae UE-ME, grown in the presence of 25, 50 and 100 μM isoproturon, shows an adaptive response to stress, stabilizing the reducing cytoplasmic environment and a decrease of MDA cytoplasmic content. In addition it was observed an adaptively attenuated activation of GR and GPx activities for 25, 50 and 100 μM isoproturon, in the culture medium. Although there will be a decrease of intracellular NADPH/NADP⁺ ratio, due a decrease of G6PD enzyme activity, this effect seems not sufficient to block responses mediated by antioxidative cycle of glutathione, which has been above discussed, probably due to a decrease of the proportion of apoptotic dying S. cerevisiae UE-ME. Having regard to the mentioned above, we can presume that S. cerevisiae UE-ME reveal high resistance to isoproturon. If further studies confirm the ability of this strain to use isoproturon as carbon or nitrogen source is possible to use this strain in bioremediation processes.

Keywords: Sacharomyces cerevisiae, isoproturon, lipid peroxidation

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Seasonal Monitoring of the Microbial and Physico-Chemical Quality of Two Rivers in Durban, South Africa

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Recently, concerns over water quality have increased globally due to frequent contamination of coastal and inland water resources by water-borne microbial pathogens and chemical pollutants. Many inhabitants of informal settlements in South Africa rely on surface water sources for their daily water needs due to lack of access to potable water. In this study, the effects of seasonal changes on the microbial and physico-chemical quality of the Umgeni and Umdloti rivers in Durban, South Africa were evaluated. Microbial analysis was conducted using membrane filtration techniques and subsequent plating on selective media. Heavy metals were analyzed using ICP-OES, and other physico-chemical parameters determined using standard methods for the examination of water and wastewater. Both river water samples analyzed in this study were poor in microbial quality throughout the seasons, with highest coliform counts observed during summer and the lowest during winter. Total coliform and faecal coliform populations varied between 1.468 x 10^2 and 7.4 x 10^2 cfu/100ml, respectively in both river samples. Similarly, faecal Streptococci populations varied between 0.5 – 17.55 x 10^2 cfu/100ml across the various sampling points, with high Enterococci populations also detected at some points. Of the physico-chemical parameters analyzed, only pH, temperature and aluminium fell within the acceptable limits while the levels of turbidity, BOD, COD, lead, mercury, cadmium and copper all exceeded their limits on all occasions throughout the seasons. Heavy metal contamination of the surface water resources in Durban. This could pose health risks to the users of these waters and emphasizes the need for implementation of improved management strategies of these river catchments for continued environmental sustainability.

Keywords: Coliforms; E. coli; River water; Seasonal variation; Heavy metals

Simultaneous degradation of atrazine and simazine by Arthrobacter sp and Stenothrophomonas sp., in a packed bed reactor.


Triazine compounds are a group of widely used herbicides. From these, at least atrazine, simazine and cyanazine have been reported to be toxic for aquatic systems. Frequently, water bodies such as ponds and creeks receiving pluvial drainage from agricultural soils are contaminated by toxic compounds. The use of biological barriers containing microorganisms able to degrade these pesticides could reduce their harmful effects to the susceptible aquatic biota.

In this work, the simultaneous biodegradation of a mixture of atrazine and simazine by a binary community constituted by Arthrobacter sp. and Stenothrophomonas sp., immobilized in a porous support is reported. Both strains are able to degrade these herbicides. Atrazine, simazine and cyanazine are biodegraded by a mixture of Arthrobacter sp. and Stenothrophomonas sp., immobilized in a porous support. Both strains are able to degrade these herbicides. Atrazine, simazine and cyanazine are biodegraded by a mixture of Arthrobacter sp. and Stenothrophomonas sp., immobilized in a porous support. Both strains are able to degrade these herbicides. Atrazine, simazine and cyanazine are biodegraded by a mixture of Arthrobacter sp. and Stenothrophomonas sp., immobilized in a porous support. Both strains are able to degrade these herbicides. Atrazine, simazine and cyanazine are biodegraded by a mixture of Arthrobacter sp. and Stenothrophomonas sp., immobilized in a porous support. Both strains are able to degrade these herbicides.

To degrade the herbicides mixture (80% atrazine and 20% simazine, in mineral salts [MS-AS] medium), a 2.86 L two-stage column packed with fragments of porous volcanic rock (tezontle) was used. Both reactor stages have a porous glass plate for air distribution. Air and MS-AS medium were concurrently fed to the biofilm reactor. The system was continuously operated varying the triazinic loading rates (R_{V,AS}) from 0.127 to 0.53 mg L^{-1} d^{-1}, obtaining removal efficiencies (\eta_{AS}) from 92 to 100%, measured as Chemical Oxygen Demand ([COD]; Method 8000, Hach, 2002), and 91 to 97 %, quantified as Total Organic Carbon ([TOC]; Method 10129, Hach, 2002). These last values were similar to those obtained by liquid chromatography (HPLC).

Keywords: biodegradation; herbicide, biofilm, packed bed reactor, atrazine, simazine
Simultaneous biological removal of ammonia nitrogen, phenol and formaldehyde from high-concentration wastewaters using an MSCR system

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A novel moving-bed sequential continuous-inflow reactor (MSCR) was developed and investigated for the degradation of high concentrations of ammonia nitrogen, phenol and formaldehyde. An aerobic process of simultaneous heterotrophic organic and direct autotrophic nitrogen-removal (SHODAN) was observed in the MSCR. Results indicated the bioprocess could simultaneously remove greater than 99% of the target compounds for concentrations up to 1300 mg/L each and around 96% of the corresponding chemical oxygen demand (COD) of ~4800 mg/L with a 6-h cycle time. An increase of the inlet concentrations to 1500 mg/L, however, caused a slight reduction in the removal efficiency. The MSCR handled hydraulic shock loads of up to four times the normal flow rate without adversely affecting the elimination performance of the contaminants. These unique features, combined with the efficient and compact nature of the process, thus recommend MSCR as a very promising technique for the simultaneous removal of nitrogen and organic compounds in a single-basin bioreactor.

Keyword: bioreactor, moving bed, activated sludge, ammonia nitrogen, organic compounds.

Soil Microbes and their Beneficial Roles to Improve the Environmental Quality

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Microbes are everywhere in the biosphere, and their presence invariably affects the environment that they are growing in. The effects of microorganisms on their environment can be beneficial or harmful or invisible with regard to human measure or observation. The beneficial effects of microbes derive from their metabolic activities in the environment, their associations with soil, plants and animals, and from their use in food production and biotechnological processes. Decomposition or biodegradation results in the breakdown of complex organic materials to forms of carbon that can be used by other organisms. There is no natural organic compound that can not be degraded by some microbes, although some synthetic compounds are broken down slowly or not at all. Since microbes have been present on the environment longer than other organisms, they have evolved the ability to thrive in almost any environment that meets these minimal criteria. In one handful of healthy soil there are literally hundreds of species of soil bacteria, soil fungi and many other microscopic soil microorganisms. The major role of soil microbes is to decompose organic materials around the root zone or soil body and releases some organically bound nutrients into inorganic forms. Nowadays, there is an increasing awareness of the importance of soil microbes amongst growers. Producing available nutrients to plants and acting as a biofertilizer are just some of the tens of their beneficial roles in the soil media. Soil bacteria will actually reduce soil compaction by improving soil structure creating microscopic spaces or pores in the soil to hold air or water. Some other soil bacteria act as a hunter and will suppress soil pathogens that could cause disease in some plants, reducing the need to use pesticides. The use of beneficial natural microbes to treat different environmental pollution and sickness and stimulate the environment is an ancient and proven technology dating back thousands of years. Natural soil microbes have been added to water, soil, sewage and oil spills and other such media to improve their quality and cleanup them through bioremediation processes and have many other uses. Microbial products effectively reduce odors and nutrients in water and are proven to improve plant and soil health in agriculture as well as in the environment. This paper is trying to explain the environmentally beneficial role of soil microbes acting as useful organisms to significantly improve the soil and environmental quality.

Keywords: soil microbes, environment, biofertilizer, bioremediation.
Structure of bacterial consortia in glacier lagoons (King George Island, Antarctica)

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Microbial communities were investigated in the areas abandoned by retreating glaciers at the western shore of Admiralty Bay (King George Island, Antarctica). Process of deglaciation open a new areas for colonization by both autotrophic and heterotrophic microorganisms. Decreasing of salinity of the water in glacial lagoons is a main factor of the halophilic organisms mortality. The meltwater runoff from the glacier and the streams from lateral moraines as well as seaweeds – deposited on shore and than transported by wind to new ice-free areas are considered as a potential source of microorganisms at the deglaciated areas. Microbiological process of decomposition and mineralization of organic matter play key role in nutrients enrichment of these areas. In the study, variation in the bacterial community structure of three glacial lagoons was determined. Samples were collected from water, bottom sediments, algae periphyton and the surface of stones (shore zone of lagoons) at the front of glaciers: Ecology, Baranowski and Windy. Bacterial community structure was determined using a combination of PCR amplification of 16S rRNA gene fragments and denaturing gradient gel electrophoresis (DGGE). The CTC and Live/Dead staining methods were used to detect metabolic active bacteria. Results indicated marked changes in the bacterial community structure. Comparison of the results suggest that increased levels of nutrient input in shore zone of lagoons and bottom sediments will lead to marked changes in the structure and physiological activity of the bacterial consortia.

Keywords: Antarctica, bacterial community structure, deglaciation, DGGE.

Structures, activities and biosynthesis of cyanobacterial peptides

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Cyanobacteria produce a wide variety of small linear and cyclic peptides. One group of the best known peptides are the cyanobacterial hepatotoxins, microcystins and nodularins. Mass occurrences of toxic cyanobacteria have caused a number of animal poisonings and pose a risk for human health. Cyanobacterial hepatotoxins are produced nonribosomally by large multi-enzyme complexes which contain both nonribosomal peptide synthetases (NRPS) and polyketide syntheses (PKS) as well as tailoring enzymes. We showed that the hepatopidine nosothycin which bears structurally similarities to microcystins is encoded in a 45-kb NRPS gene cluster. Cyclic anabaenopeptolides (cyanopeptolins) and anabaenopeptins as well as the linear peptides spumigins were found to be products of NRPS. These compounds are protease inhibitors. In anabaenopeptin biosynthesis a new way to create non-ribosomal peptide structural diversity was detected. Newly discovered cyanobacterial bioactive compounds in our laboratory include peptides which act as antidotes for microcystins and lipopeptides destroying the eukaryotic cell membrane. The genome project of Anabaena strain 90 led us to discover ribosomal pathway to produce cyclic peptides, cyanobactins. We demonstrated the widespread (48 out of 132 strains) but sporadic occurrence of the cyanobactin biosynthetic pathway among planktonic cyanobacteria by PCR. Cyanobactins termed as anacyclamides characterized by LC-MS from 29 Anabaena strains showed great length (7-20 amino acids) and sequence variation (only proline was common in all anacyclamides). The identified anacyclamides comprised of unmodified proteinogenic (not previously detected in cyanobacteria) or prenylated amino acids. Cyanobacteria produce peptides by ribosomal and nonribosomal pathways. Many of these cyanobacterial compounds may be of interest for pharmaceutical industry or find their way as cell reagents. In addition, their biosynthetic machineries (ribosomal and nonribosomal) provide enzymes to be used in combinatorial biosynthesis or chemoenzymatic synthesis to produce novel compounds in the future.

Keywords cyanobacteria; bioactive compounds; peptides; ribosomal and nonribosomal biosynthesis; cyanobactins

References

Studies on extremophilic *Bacillus* SB1 isolated from n-butanol enriched mangrove sediment

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Studies were carried out on *Bacillus* SB1, a unique extremophilic bacterial strain, earlier isolated from mangrove sediment of Goa, India, by a stepwise n-butanol enrichment process. SB1 exhibits high level of tolerance to various organic solvents such as decane, hexane, cyclohexane, toluene, benzene, xylene, chloroform and also to n-butanol which is considered extremely toxic. Solvent tolerance is a stable phenotypic property of SB1 hence the culture withstands solvent shock and live cells are isolated from solvent saturated media after prolonged exposure.

SB1 degrades aliphatic and aromatic hydrocarbons. It exhibits excellent growth at high temperature, high pH and high salt concentrations and hence appears to be a promising candidate for bioremediation studies.

SB1 utilises cholesterol as the sole source of carbon and hence can be used in development of biphasic organic-aqueous steroid transformation systems.

Since n-butanol tolerance is a rare trait, effect of n-butanol on the cells was studied. The culture grows in upto 2% v/v n-butanol but growth is severely retarded at 3%. Presence of butanol does not stimulate sporulation, however spores germinate in concentrations allowing growth. Adherence assays reveal that the culture does not adhere to solvent but emulsifies it. Electron micrographs reveal that cells grown on butanol saturated plates are shorter in length as compared to cells grown without butanol exposure.

**Keywords** organic solvent tolerance, n-butanol, cholesterol

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Study of photocatalysis as a pre-treatment for azo dyes removal

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The widespread use of dyes in the world leads to large amounts of coloured wastewater. Owing to their coloration, the dyes cause floral pollution and aesthetic pollution. Some dyes especially azo dyes are also potentially toxic and their removal is a great challenge for the scientific community.

Azo-dyes are few or not biodegradable and a sole biological treatment, the lowest cost process, cannot be considered for a purification of textile effluents.

To remove recalcitrant, inhibitory or toxic compounds for microorganisms, integrated processes could be an effective solution, more especially the coupling of advanced oxidative processes and a biological treatment. An advanced oxidative process can be implemented as a pre-treatment in order to increase the biodegradability of the polluted effluent, more readily assimilable by microorganisms.

The aim of this study dealt with the feasibility of coupling photocatalysis and a biological treatment for the removal of azo dyes from aqueous effluents. During photocatalysis pre-treatment, a decrease of the Chemical Oxygen Demand showed an oxidation of the target compound and by products i.e. a global change in the chemical structure of the complex mixture. A higher biodegradability is expected for high level of compounds oxidation However, the concomitant decrease of the Dissolved Organic Carbon, characteristic of a high mineralization yield, led to nearly constant COD / DOC ratios, which was unfavorable to an increase of the biodegradability, and was confirmed by the low values found for the ratios Biological Oxygen Demand (BOD\(_5\))/COD, which remained in the range 0.09 – 0.19, namely below 0.4 after photocatalytic reaction. Moreover, toxicity increased or remained at a high level after irradiation for 3 h of the considered azo dyes, and decreased only for Orange II from toxic (EC\(_{50}\) = 53 %) to moderately toxic (EC\(_{50}\) = 76 %). An integrated process involving photocatalysis and biological treatment to treat azo dyes appeared therefore, and in the tested conditions, not really relevant, except for Orange II. More specific pre-treatment, namely involving less reactive species than hydroxyl radicals, should be promoted to yield a more favorable COD / DOC ratio.

**Keywords**: Photocatalysis; Azo dyes; Kinetics; Biodegradability; Toxicity.
Study on efficiency of activated sludge system by using fireclay as sorbent

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Abstract

Background and aims: Addition of mineral particulates into the aeration tank in the activated sludge system will lead to increase in bacterial population and subsequent increase in organic removal efficiency because of attachment and growth of bacteria on to particulates surfaces. Also attached bacteria have more tolerance potential to toxic shocks entering the system. If the added material in the aeration tank has sorption capacity, the toxic compounds harmful to microbial community can be removed and the system efficiency will be enhanced. The aim of this study was determination the efficiency of activated sludge system by using the fireclay as additive material.

Methods: Experiments were conducted by using an activated sludge system in bench scale operating in batch and continuous modes. Artificial wastewater was used as model domestic wastewater. In the continuous mode, hydraulic detention time in the aeration reactor was 8 and 22h. In the batch mode, aeration time was 8, 16 and 24h. Fire clay doses were 500, 1400 and 2250 mgL$^{-1}$ and was added into the reactor in each experiment separately. Efficiency parameters such as BOD, COD, MLVSS and Nitrate were measured according to standard methods before and after of addition of fireclay in the system separately.

Results: The average efficiency for COD removal before and after addition of fireclay was 55% and 95% respectively. The average concentration of volatile suspended solids (bacterial biomass) before and after addition of fireclay was 2210 and 4000 mgL$^{-1}$ respectively. By using fireclay as additive to activated sludge system, nitrification was enhanced and concentration of nitrate was increased by 80%. Increasing the fireclay dose, will cause increase in system efficiency.

Conclusion: Addition of fireclay in the activated sludge will enhance the efficiency of system.

Keywords: fireclay, activated sludge, efficiency

The Bacterial Consortium Alleviated a Low-dose Gamma-Irradiation in Kalanchoe Plantlets


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Ionizing radiation has been used to study role of bacterial association in protection of Kalanchoe daigremontiana plantlets from its hazardous effect. Two defined plant growth promoting bacterial strains were used for inoculation of plantlets before acute irradiation with $\gamma$-quanta ($^{60}\text{Co}$). Lethal dose of the $\gamma$-rays for Klebsiella oxytoca IMBG26 was 3.0 kGy, and for Paenibacillus sp. IMBG156 it was 800 Gy. K. oxytoca IMBG26 expressed enhanced activity of the pelX promoter after a sublethal dose of irradiation. The pelX promoter activity measured as activity of $\beta$-galactosidase of the pelX(::lacZ) fusion in K. oxytoca (pGalP) and was 0.88 mkM/ml·min after exposure to 2.0 kGy, e.a. 80% of control (untreated) bacterial activity, although the irradiated bacterial population comprised 1.25% from control one. Integrated index (II) of plantlet development which relied on both root number and root length reflected fluctuations in metabolic processes in irradiated plantlets without treatment with bacteria. Stabilizing stress-reactions occurred on 10 days post irradiation at different doses (30, 50, 70 Gy), however, II remained at the level of 30-60% to control plantlets. Effect of irradiation on kalanchoe plantlets relieved by bacteria at 30 and 50 Gy, moreover, II was observed at levels of 500 and 200%, respectively. The adaptive response was evident after both doses given for K. daigremontiana plantlets. Intense root elongation, instead of development of new coronal roots, led to fast adaptation to stressful conditions and normalization of metabolic processes in kalanchoe plantlets. We consider ionizing-radiation-resistant bacteria may be used as a protection against ionizing radiation damage in sensitive plants.
The diversity and distribution of sulfate reducing microorganisms in a high-temperature oil reservoir

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Injection of seawater to subsurface oil reservoirs is routinely performed in the oil production in order to maintain reservoir pressure and increase oil yield. The introduction of surface water dramatically stimulates the growth and activity of microorganisms in the reservoir, creating a large-scale enrichment culture of subsurface anaerobic microorganisms. Due to the extreme conditions in the reservoirs (high temperature, salinity, and pressure), these oil reservoirs constitute highly interesting systems for studies of subsurface extremophilic microorganisms. To the industry, the sulfate-reducing microorganisms - both Bacteria (SRB) and Archaea (SRA) - are particularly interesting because they contribute to reservoir souring (sulfide production) and increased rates of corrosion on steel surfaces in the production system. To minimize the detrimental effects of sulfate reduction, the industry expends considerable resources on mitigation measures, such as addition of nitrate to injection water, biocide treatment, mechanical cleaning of metal surfaces, etc.

In this study, the diversity of sulfate-reducing microorganisms in high temperature (about 80ºC) oil reservoirs from the North Sea and the associated oil production system was determined by gene cloning and sequencing. Several groups of SRB affiliated with Deltaproteobacteria, Firmicutes and other, deeply rooted and less well-described groups were found. SRA affiliated with the genus Archaeoglobus were also widespread in the system. Customized primer sets for qPCR-based enumeration of the dsr genes of the detected sulphate reducers were developed and used to survey their distribution in the reservoir and production system in relation to environmental parameters such as in situ temperature and injection water characteristics. The results indicated that the high-temperature parts of the system were dominated by SRA whereas the abundance and diversity of SRB increased dramatically with decreasing temperature. The effect of nitrate addition to injection water will be discussed in relation to the observed dominance of Archaeoglobus in the reservoir and its possible reduction of nitrate. Furthermore, the perspectives of using commercially available molecular microbiology tools in future surveillance of oil production systems will be evaluated.
The effect of sodium selenite and selenate on the quality of lettuce and soil microbiological activity

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Selenium is considered to be essential element for humans, animals and some species of microorganisms. In human and animal cells Se plays an essential role in antioxidative defense system, but it is toxic at high dietary intake. In many countries soils are low in bioavailable Se. Selenium enters the food chain through the plants which take it up from soil. Se concentration in plants depends on the chemical form of Se, its concentration and bioavailability in soil and soil microorganisms. The aim of the study was to detect the effect of sodium selenite and selenate on the soil microbial activity and physiological properties of lettuce.

Two varieties of lettuce plants (Lactuca sativa): iceberg lettuce ‘Tarzan’ and lettuce ‘Rīga’ were grown in 1L pots and during growth season were once treated with 50 mg m\(^{-2}\), 100 mg m\(^{-2}\) or 200 mg m\(^{-2}\) of sodium selenite or selenate. Control - without treatment. Fresh and dry weight of lettuce leaves, pigment content and antiradical activity were tested three times during vegetation period. Soil respiration was determined by ADC 2250 Gas analyzer.

The higher soil respiration was observed in the soils treated with selenium. Soil microbial activity depends on used lettuce variety- soil where iceberg lettuce ‘Tarzan’ was grown has less activity in comparison with ‘Rīga’ one.

Key words: lettuce, soil respiration, selenite, selenate

The role of fungi in the oxalate-carbonate pathway

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The oxalate-carbonate pathway has been studied in acidic, tropical soils and involves plants, fungi, and bacteria. It consists of a biomineralization process followed by accumulation of calcium carbonate (CaCO\(_3\)). The atmospheric carbon (CO\(_2\)) is sequestered in the plant biomass through photosynthesis and is partly accumulated as calcium oxalate (CaOx). Fungi are also CaOx producers. Saprophytic fungi assimilate the carbon of plant litter and use part of it to synthesize oxalic acid, which spontaneously forms highly stable CaOx. Furthermore, fungi decay CaOx-containing plant webs in the litter, exposing this salt to microbial decay. Although highly oxidized, CaOx is an efficient substrate used by oxalotrophic bacteria as an energy and carbon source, and consequently does not accumulate in the geological record. Half of the carbon contained in CaOx is transferred to the soil in the form of calcium carbonate during bacterial oxalate catalolysis. Finally, CaCO\(_3\) accumulates in the soil.

Understanding the role of fungi in the oxalate-carbonate pathway necessitates in-depth study of fungal interaction with minerals, bacteria, and plants.

Fungal CaOx crystals are constituted by oxalic acid, which is produced by the fungus, and calcium, which is either present in the environment solution, or secreted by the fungus itself. CaOx formation may simply be due to the presence of calcium in the environment. However, a calcium efflux exists at the hyphal tips, but it is not known if fungi control the concomitant efflux of calcium and oxalic acid to form CaOx. Oxalic acid is released by a large number of metabolically active fungi. In our study, 21 out of 25 decay Basidiomycetes were CaOx producers. In vitro experiments have been conducted to trace the calcium source contributing to CaOx crystal precipitation: calcium is transferred along the mycelial network, transported, and liberated from distant regions during cell lysis or due to active mechanisms that still must be elucidated. Our study provides insight into the ability of fungi to consume CaOx: the abundance of the crystals in the medium decreased within a few days (Fig. 1), bringing into question the assimilation of CaOx by fungi.

These studies have been conducted first with axenic cultures and the next step will be to get closer to field conditions. This evolutionary path followed by fungi cannot be dissociated from the bacteria that share their habitat. CaOx is a very common and widespread compound and it is likely to be competed for by bacteria and fungi. It is crucial to consider both types of organisms acting together to elucidate their respective roles.

In vitro experiments with microcosms have been performed to bridge the gap between field observations of the oxalate-carbonate pathway and its in vitro occurrence. We reconstituted the oxalate-carbonate pathway in controlled laboratory conditions by combining selected bacterial and fungal strains. Microcosms, containing sterile acidic soil with the addition of straw, have been injected with bacteria only, fungi only, and a combination of both, and their evolution has been recorded over 3 months. Oxalate, the key initial substrate of this pathway, was added or absent (Fig. 2). pH and oxalate concentration have been monitored to understand the role of bacteria and fungi in the evolution and soil colonization. The relative importance of organisms in the variation of parameters was assessed by measuring both the fungal biomass (ergosterol) and the bacterial abundance (Q-PCR).

Fungal and bacterial participation in the oxalate-carbonate pathway is not yet fully understood but new insights are expected from microcosm analysis and electron microscopy observation in the distribution of bacteria, fungi, and calcium oxalate crystals.

Keywords oxalate-carbonate pathway; fungal calcium translocation; fungal-bacterial interaction; Australian soil

Figure 1. Pycnoporus cinnabarinus, calcium oxalate crystal production and consumption (CaOx counted visually)

Figure 2. A simplified microcosm and the combination tested (in the frame). Ox = oxalate
The role of salinity in shaping inorganic nitrogen and N$_2$O dynamics in estuarine sediments.

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Nitrogen (N) is a key determinant of the functionality of estuarine ecosystems since it can limit biological growth and consequently be implicated in eutrophication processes. On the other hand, estuaries by nature are dynamic systems exhibiting a high degree of temporal and spatial salinity variability. In this study, the role of salinity estuarine gradients on sediment inorganic nitrogen fluxes was investigated in three NW Portuguese estuaries (Douro, Ave and Câvado). Sediment was sampled at the shallow and upper sections of each estuary. Shurry experiments were run anaerobically at salinities 0, 10 and 25 and the fluxes of nitrate, nitrite, ammonium and nitrous oxide were monitored. The salinity-induced NH$_4^+$ sediment desorption was observed for all sites. While in Douro and Câvado estuaries, no significant changes were detected on NO$_3^-$ consumption, in the industrial polluted Ave estuary samples NO$_3^-$ consumption increased 10 times when the salinity rose from 0 to 10. However, salinity appeared to have little effect on nitrite fluxes. N$_2$O production increased sharply with increasing salinity in the upper sites. Although similar stimulation of N$_2$O emissions was not observed in the lower sites, the overall production emerges as a major concern since N$_2$O is a powerful greenhouse gas. We conclude that changes in salinity have a significant effect on nitrogen dynamics in estuarine sediments. Furthermore, the trend for freshwater discharge reduction, due to water diversion for agriculture within the watersheds and climate change in these estuaries could exacerbate N$_2$O production (global warming) and NH$_4^+$ availability to primary production (eutrophication).

This work was funded by this work was supported by the Portuguese Foundation for Science and Technology (FCT), through a scholarship (POCTI/ICTA/39034/2001) and a research project (PTDC/AMB/64441/2006).

Keywords: estuaries; salinity; nitrogen; nitrous oxide

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Thermophilic bacteria isolated from a personal-use composting reactor

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Composting is an efficient way to treat organic waste without generating harmful compounds such as dioxins produced by incineration. In this processes, thermophilic and mesophilic microorganisms contribute for decomposition of complex organic substrates. To reveal their physiological properties and functions in the composting process, we tried to isolate thermophilic microorganisms from a personal-use composting reactor.

Composting reactor for household use, “Nagamomi-eater” (TK401-T, Matsushita Electric Works), was applied for composting reaction. The working volume of the reactor was 15 L, and the temperature was usually maintained above 40°C and reached about 60°C during the active degradation of organic matter. The biomass carrier is comprised of 5 L (1.2 kg wet wt) of wood chips (about 0.5 - 2.0 mm). The artificial organic waste of 500 g of dog food containing 80% (w/w) water was loaded daily into the reactor. The contents were gently mixed twice per minute by automated paddles. The sample for the isolation of microorganism was taken from the optimal conditioned reactor in which the rate of decomposing organic matter was about 17 g L$^{-1}$ day$^{-1}$. The temperature and pH of the sample were 47°C and 8.6, respectively. The diluted-sample was plated onto the modified Brock’s basal salts (Kurosawa et al., 1998) supplemented with 0.2 % (w/v) yeast extract, pH 7.5, solidified by 0.7 % (w/v) Gelrite, and incubated at 60°C for 24 h. We have also tried enrichment cultivation by using liquid media at 60°C and 70°C for 24 h prior to the plating. Colonies appeared on the plates were purified and their genomic DNAs were extracted for PCR amplification of 16S rNA genes (16S rDNA) using bacterial universal primers. The following thermal cycle was used for 25 cycles: 95°C for 30 s, 60°C for 30 s, and 72°C for 1.5 min. DNA sequencing was carried out by using automated DNA sequencer. The obtained 16S rDNA sequences were compared with the available sequences in the NCBI database using BLAST. The 16S rDNA sequences of the isolates and relative species were aligned using Clustal W program. Phylogenetic trees were constructed using the neighbor-joining method.

Total 11 strains were successfully purified and taxonomically classified into four species. They showed 16S rDNA similarities with *Ureibacillus thermosphaericus* (similarity value of 98%, 1 strain), *Geobacillus thermoglucosidasius* (97-99%, 6 strains), *G. toebii* (99%, 3 strains) and *Thermobacillus* sp. (96%, 1 strain). Among these isolate, strain KWC4, showed a 96 % of 16S rDNA sequence similarity with *T. xylanilyticus*, which had MK-7 as the major. The fatty acid composition of these strains were also different each other. On the basis of phenotypic and genotypic evidences, the strain KWC4 represents a new species, for which the name *T. composti* sp. nov. was proposed (Watanabe et al., 2007). *T. composti* showed xylanase activity. Analysis of its enzymatic properties and cloning of the xylanase gene are now in progress.

Acknowledgement

This work was supported by “The Next-generation Joint Research” Project for fostering of young researchers: matching fund subsidy from Soka University, Japan, 2009-2010.

References


Keywords: thermophilic bacteria; compost; novel species; xylanase
Thermophilic Co-Digestion of Cellulose and Microalgal Biomass for Hydrogen Production

Sarah M. Carver, Chris Hulatt, David N. Thomas, and Olli H. Tuovinen

Potential commercial-scale production of biodiesel from microalgal biomass has generated a great deal of research and development due to climate change and renewable energy resource policies. Anaerobic digestion of mass microalgal harvest or spent biomass following lipid extraction has prospects for other forms of energy generation, but research in these areas has been limited to date. In this study, untreated biomass of two microalgal species, *Chlorella vulgaris* and *Dunaliella tertiolecta*, were used as co-substrates with cellulose and the mixtures were incubated at 60°C anaerobically with a cellulolytic consortium. After several enrichments, the cultures were monitored for head space gas composition, short chain fatty acids, and C:N ratios. The results of gas analysis showed that maximum H$_2$ yields, 9 mmol g·VS$^{-1}$, were obtained with a 1:2 wt/wt ratio of *D. tertiolecta* biomass to cellulose. The reference cultures containing only cellulose yielded 6 mmol H$_2$ g·VS$^{-1}$. *C. vulgaris* showed relatively little H$_2$ or CO$_2$ production with yields less than 5 mmol g·VS$^{-1}$ and 3 mmol g·VS$^{-1}$, respectively. Methane production was not detected in these experiments. Short chain fatty acid analysis revealed the cellulolytic culture to produce mainly lactic acid, 20 mM, when fed only cellulose. In the presence of *D. tertiolecta* biomass, lactic acid production decreased at least eight fold. With the presence of *C. vulgaris*, up to 40 mM butyric acid was produced, indicating a change in the consortium’s metabolic activity. Cultures grown with *D. tertiolecta* and cellulose showed a drastic decrease in the C:N ratio, suggesting that the algal biomass promoted better growth of the consortium as compared to cellulose as the sole substrate. This was also evidence that algal biomass was digested by the anaerobic consortium. Cultures containing *C. vulgaris* showed no significant change in the C:N ratio after ten days of incubation. The results indicated that *D. tertiolecta* provided a better co-substrate than *C. vulgaris* for hydrogen production. This difference may be related to the lack of a distinct cell wall in *D. tertiolecta*, which would otherwise serve to provide structural integrity and mechanical strength to the cell.

Ultrastuctural Behavior of *Rhodotorula mucilaginosa* Induced by the Growth in Presence of Pyrene

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The present study was carried out in order to evaluate the resistance / tolerance of an isolate of *Rhodotorula mucilaginosa*, a single-celled fungal, related to the ultrastructural behavior by the growth in pyrene, a polycyclic aromatic hydrocarbon (PAH). The organism was grown in Yeast Mold Broth (YMB) in the absence and presence of pyrene at concentrations of 0.25 mg/mL, 0.5 mg/mL and 1 mg/mL, under orbital agitation of 150 rpm and 28 °C. Samples were collected at intervals of 8, 12, 16, 20, 24, 48 and 72 hours of culture. The detection of the ultrastructural alterations was performed by the use of routine and cytochemical techniques for transmission electron microscopy. The ultrastructural analysis of *Rhodotorula mucilaginosa* using the routine technique revealed alterations in the fine structure of cells, related to the presence of vacuoles, cell wall electrondensity, number of eletromembranes and mitochondria. Cultures treated with pyrene exhibited differences on the staining pattern when compared to control cells and pyrene concentrations. Also, the cytochrome oxidase and catalase cytochemistry showed variations in the intensity and distribution of reaction products in the cell wall, cytoplasmic membrane, cytoplasm and mitochondria related to the pyrene concentration used during cellular growth. In the experimental conditions used in this work the presence of pyrene induced different effects on *Rhodotorula mucilaginosa* ultrastructure, which are presented for the first time in the literature.

Keywords: *Rhodotorula mucilaginosa*; Pyrene; Ultrastructure.
Use of hydrolysates from silver carp (*Hypophthalmichthys molitrix*) head as a peptone for *Vibrio anguillarum* and optimization using Central Composite Design (CCD) and Response Surface Method (RSM)

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The objective of present study was to peptone production, by enzymatic hydrolysis, with Alcalase of silver carp (*Hypophthalmichthys molitrix*) head waste. The fish peptone produced was used instead of the standard peptones which applied in commercial Tryptic Soy broth (TSB) media for *Vibrio anguillarum*. Peptones produced Alcalase had a 63.42% degree of hydrolysis. Optimization of the used peptone as culture media was also done using Central Composite Design (CCD) and Response Surface Method (RSM). Batch experiments were conducted to monitor *Vibrio anguillarum* growth for duration of 24 hours. A full factorial Central Composite Design of experiments was used to construct second-order response surfaces with the bacterium growth as dependence parameter. The head peptone concentration and the retention time were used as design factors. Analysis of variance showed a high coefficient of determination value (0.930). It was possible therefore; to develop the empirical equations describing and predicting the optimum value. Results also were shown that enzymatic-modified fish by-products can be used as low cost nitrogen sources for bacterial growth.

**Keywords**: silver carp; peptone; Alcalase; Response surface method; central composite design

Utilization and pretreatment of dairy industry wastewater by *Candida bombicola* for the production of sophorolipids

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Dairy industry is one of the major food industries in many countries including India. In dairy industries, water is a key processing medium that is used throughout the industry for several operations including cleaning, sanitization, heating, cooling, floor washing, which generates large amount of wastewater. These wastewaters contain high levels of dissolved or suspended solids including fats, oils and grease, nutrients such as ammonia or minerals and phosphates that makes them not easily biodegradable, and therefore cause gross pollution of land and water due to their high biochemical oxygen demand (BOD) and chemical oxygen demand (COD). Also, the high content of fats and oils often interfere with normal wastewater treatment procedure resulting in significant increase in the process cost and time and therefore require pretreatment before subjecting to biological treatment. Utilization of dairy wastewater for simultaneous production of bio-products is one possible way of treating this type of wastewater.

Therefore, the objective of the present study was to test the feasibility of pretreating dairy wastewater by in situ production of sophorolipids (SLs) using *C. bombicola* to reduce its biological load and to reduce SLs production cost. SLs are glycolipids type of biosurfactant and produced by the yeast *Candida bombicola*. SLs have found several pharmaceutical, medical and environmental applications, besides being useful in the formulation of detergents and cosmetics.

Dairy industry wastewater, obtained from a local dairy in Guwahati, India, was initially characterized and used as a medium, with or without external carbon and nitrogen sources, for production of SLs by the yeast *C. bombicola* (Starmerella bombicola). Different media compositions used in the study along with the results obtained was presented in Table 1.

<table>
<thead>
<tr>
<th>Medium type</th>
<th>Compositions SLs yield (g/l) COD removal efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 1</td>
<td>DW* + glucose (10%) + yeast extract (1%) + urea (0.1%) 2.8 ± 0.79 82.1</td>
</tr>
<tr>
<td>Type 2</td>
<td>DW* + glucose (10%) + yeast extract (1%) + urea (0.1%) + soybean oil (10%) 13.5 ± 0.57 99.5</td>
</tr>
<tr>
<td>Type 3</td>
<td>DW* + glucose (10%) + yeast extract (1%) + soybean oil (10%) 17.5 ± 1.4 99.5</td>
</tr>
<tr>
<td>Type 4</td>
<td>DW* + lactose (10%) + yeast extract (1%) + urea (0.1%) + soybean oil (10%) 7.5 ± 1.38 63</td>
</tr>
<tr>
<td>Type 5</td>
<td>DW* + lactose (10%) + yeast extract (1%) + soybean oil (10%) 9.4 ± 0.55 5.0</td>
</tr>
<tr>
<td>Type 6</td>
<td>DW* + glucose (10%) + yeast extract (1%) + soybean oil (10%) 62 ± 0.65 85.7</td>
</tr>
<tr>
<td>Type 7</td>
<td>DW* + sucrose (10%) + yeast extract (0.2%) + soybean oil (10%) 27.4 ± 1.6 84.0</td>
</tr>
</tbody>
</table>

*DW* = dairy wastewater added to make up the required volume

The results of this study, conducted in batch shake flasks showed that using the wastewater supplemented with extra carbon and nitrogen source, the yeast can better produce SLs and also reduce COD of the wastewater. And a maximum SLs yield of 62 g/l was obtained when the dairy wastewater was supplemented with glucose, yeast extract and soybean oil at 100 g/l, 2 g/l and 100 g/l, respectively. On the other hand, a maximum SLs yield of only 2.8 g/l was obtained with the dairy wastewater without adding extra carbon and nitrogen source. These results clearly reveal that dairy wastewater can be used in SLs production, and the amount of the biosurfactant can be satisfactorily increased by supplying extra carbon and nitrogen sources. Similarly, high COD removal efficiency was obtained when extra carbon and nitrogen source was added in the wastewater, except medium type 4 and 5, where lactose was used a hydrophilic carbon source.

High yield of SLs together with high COD removal efficiency of the yeast in the present study revealed good potential of the system in pretreating such wastewater and for production of valuable bioproducts.

**Keywords**: Dairy industry wastewater; sophorolipids; fat and oil; *C. bombicola*.
Vanadium pentoxide: an oxidative stress agent which disturbs glutathione conjugates metabolism in *Saccharomyces cerevisiae* UE-ME3

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Vanadium, atomic number 23, can be found in the nature in different oxidation states, which can range from -1 to +5. In physiological conditions V(III) and V(V) species are dominant. Though V is an oligoelement indispensable to the growth of many organisms, it is toxic when present in compounds where show oxidation status +5. Consequently, the main purpose of this study was to use the Alentejo wine yeast *Saccharomyces cerevisiae* UE-ME3 as eukaryotic model to evaluate the response to the V2O5.

Yeast cells at exponential phase were harvested and inoculated in YEPE medium with 2% (w/v) of glucose and allowed to grow in the absence or presence of V2O5 ranging from 0.5 to 2.0 mM, during 72 h. The cultures were used to obtain the post-12000 g supernatant, which was used for GSH, GSSG and MDA contents determination by fluorimetric methods according to Hissin (1976) and Durfinova (2007), respectively, and GR, GPx, G6PD and γ-GT enzymatic activities determination according to Goldberg e Sponer (1987), Flohé (1984), Postma (1989), Szasz (1976), respectively. GSH, GSSG and MDA contents as well as enzymatic activities were compared by ANOVA one-way, followed by Duncan test to identify significant differences (p<0.01).

The obtained results show a decrease of GSH/GSSG ratio, as well as, an increase of cytoplasmatic MDA level in *S. cerevisiae* UE-ME3 cells exposed to V2O5, response which point us that cell environment became more oxidant and cell damages occur when yeast growing in presence of vanadium (+5). In other hand, it was observed an increase of GR activity, in all V2O5 concentration tested in this study, and an increase of GPx activity in yeast cells exposed to 1.5 and 2.0 mM V2O5. However this apparent anti-stress response mechanism to the V2O5, via glutathione redox cycle, should be addressed to S. H. Lee (sangheelee@mju.ac.kr) and J.-H. Lee (jlee@kordi.re.kr).

Keywords: *Saccharomyces cerevisiae*, vanadium pentoxide; oxidative stress

References


Vertical profile of bacterial community in the sediment of Ulleung Basin: Implication of the presence of methane-driven community

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Herein, we describe phylogenetic analysis of the bacterial community in the deep marine sediment collected at the south-western archibental zone of Ulleung Basin, East Sea of Korea (35.77° N, 130.03° E). The 780 cm deep sediment sample (GHT core) was collected by piston-coring. The core was divided into three portions; top, middle, and bottom by depth (GHT: 4-20 cmbsf-cm below of Seafloor; GHM: 380-400 cmbsf; and GHB: 660-680 cmbsf), and subjected to molecular diversity analysis based on SSU rDNA. Phylogenetic analyses revealed that over 99% of sediment clones from a total 177 clones were similar to uncultured environmental clones. The most abundant phylogenetic group in sediment core was candidate division JS1 whose proportion profoundly increased with depth and that seemed to participate the methane oxidation process. The members of the *Deltaproteobacteria* containing sulfate-reduction bacteria were abundant in the middle layer of the core. A few clones were affiliated into the *Firmicutes, Fusobacteria, Acidobacteria*, and candidate divisions GN, OD1, TM6 and WS, reflecting rich bacterial diversity in the Southern marginal region of Ulleung Basin. Conclusively, deep sediments of the Ulleung Basin retained a vast amount of uncultured bacterial diversity, which was similar to those of anaerobic sediment, soil in the methane hydrate, or sulfate related sites. The deep profile of microbial diversity suggested that microbial populations in the study seemed to be affected by the presence of methane gas in the deep layer of sediments. Correspondence should be addressed to S. H. Lee (sangheelee@ijn.ac.kr) and J.-H. Lee (jlee@kordi.re.kr).

Keywords: deep marine sediment; molecular diversity; methane-driven community
Volatile Fatty Acids Separation by ion exchange chromatography in fixed bed column

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This paper studies the separation of volatile fatty acids (VFA), produced in anaerobic reactor, through ion exchange chromatography. These acids are recurrently produced and accumulated in anaerobic reactors and their recovery for later reuse, become increasingly important whereas not only the treatment of wastewater is required, but the utilization of by-products is encouraged, to cause economic and environmental benefits. Thus, studies on techniques of separation of acids, as well as the kinetics of the process are necessary for the viability of the recovery procedure. The separation technique discussed in this work is the ion exchange and aims to promote the separation of the mixture of volatile fatty acids (VFA) from the anaerobic reactor, in fractions of acetic, propionic, butyric, isobutyric and isovaleric acids, using ion exchange in a fixed bed column. To achieve this, the work includes the study of equilibrium isotherms and kinetics of adsorption and desorption for each pure acid, individually, for the mixture of pure acid and for the mixture of VFA from an anaerobic waste water treatment.

The exchange resin used in the experiments was Amberlite IRA-410 Cl, Rohm & Haas Company, which is a resin with a strong base, the reactive group is a quaternary amine and the matrix is a styrene-divinyl benzene gel. The lifting of the equilibrium isotherms was done by batch tests in shake flasks and testing nine initial concentrations ranging from 30g.L-1 to 0,1 g.L-1. The survey of kinetics of adsorption and desorption was done through batch tests with magnetic stirring, in which samples were collected from time to time. Analysis of VFA was performed by gas chromatography. The temperature for all experiments was maintained at 25 °C. The equilibrium isotherms obtained for each acid studied are shown in Figure 1, where is possible to see that each acid have different affinities for the used resin. The parameters of Langmuir isotherm were obtained by linearization of the isotherms, and showed that the acid that has a higher affinity for the resin is butyric acid and the one wich has the largest adsorption capacity of the resin is the isobutyric acid.

The kinetic tests done for each acid studied show that ion exchange occurs rapidly, indicating that the prevalence of the process should be related only to the kinetics and less limited by diffusion in the pores of the resin.

From the results obtained we can concluded that it is possible to remove the VFA present in solution, and also owing to the differences of interaction, particularly between the acetic acid and propionic and butyric acids, is possible to separate them in a fixed bed process, which will be studied further in this work.

Keywords: volatile fatty acids; ion exchange column

Yeasts from acidic aquatic environments: towards an ecological understanding

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The study of microbial diversity in extreme environments, such as acid and/or heavy metal polluted water, is important from both scientific and biotechnological points of view. The comparison of microbial communities of such environments from different geographic areas is of interest for the detection of highly adapted species, and also important for physiological and genetic studies aiming the elucidation of mechanisms that allow the colonization of such habitats. These studies may also reveal new biocatalysts, such as extremozymes, or inspire novel bioremediation strategies. We compared novel yeasts of the genus Cryptococcus inhabiting acidic aquatic environments of anthropogenic (São Domingos Mines, Portugal - MSD) and volcanic origin (Rio Agrio-Caviahue lake, Argentina - RAC). Strains were studied by conventional and molecular techniques (PCR fingerprinting, AFLP, rRNA gene sequencing), and their tolerance to heavy metals was assessed (Cd2+, Co2+, Cu2+, Li+, Ni2+ and Zn2+). On the one hand, strains of an undescribed Cryptococcus species common of both acidic environments, isolated from the most extreme conditions, were found to be phenotypically similar and with identical D1/D2 and ITS sequences. The species showed unique acidophilic characteristics and was considered the most adapted species inhabitant in both studied sites. However, when strains of MSD where exposed to RAC water, the viability of the formers was considerably lower, revealing different survival capabilities and hence physicochemical adaptations. In addition, tolerance to heavy metals was not identical between MSD and RAC strains. However, genetic differentiation of the Argentinean and Portuguese populations was only achieved when studying highly differentiating genomic fingerprinting methods as PCR-fingerprinting and Amplified Fragment Length Polymorphisms (AFLP). Considering the large geographic distances and the different histories of both acidic environments, the genetic differences between the two Cryptococcus spp. populations were low. Differences were found only after applying MSP-PCR, RAPD and AFLP techniques, known to be good genomic tools for intraspecific differentiation. The peculiar acidophilic characteristics suggest a high dependence for acidic environments and probably lead to a yet unknown specific ecological role of this species. Due to the high selection pressure imposed by the physicochemical conditions of the acidic environments studied, it is possible that this yeast species presents a much conserved genome, but further studies should be carried out to confirm this hypothesis. On the other hand, four related Cryptococcus species were compared phylogenetically because they showed interesting physiological characteristics suggesting that they were also adapted to acidic habitats. Three of them were isolated from Portugal, described as C. aciditolerans, C. ibericus and C. metalloilereus, and one from Argentina, named C. agrionensis. The latter species was the most divergent one. These species formed a well-seperated phylogenetic cluster within the Filobasidiales of the Agaricomycotina (Basidiomycota). Considering that these new species also shared a peculiar ecology, being able to thrive in extreme environmental conditions characterised by very low pH and high concentrations of heavy metals, this combination of phylogenetic and ecological characteristics was designated as an eoclae. The members of the eoclae probably share a common ancestor that bored less extreme conditions than the acidophilic species, but was also autochthonous of acidic environments.

Keywords: Cryptococcus, Eoclae, Heavy metals, Microbial ecology.
16S Ribosomal Ribonucleic Acid Analysis of Pathogenicity in Autothermal Thermophilic Aerobic Digestion Treated Swine Manure

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Antibiotics are used excessively in livestock farms for infection treatment, prophylaxis, and nutrient. Such excess prescription could leads to emergence antibiotic resistance in bacteria. The emerged resistance mechanism can be transferred to pathogens giving them antibiotic resistance. Therefore swine manure could be a reservoir for antibiotic resistant pathogens. In previous study, antibiotic resistance and pathogenicity of swine manure (LM), lagoon fermented swine manure (LF) and Autothermal Thermophilic Aerobic Digestion (ATAD) treated swine manure (LA) were assessed using culture dependent method. The result have revealed large quantities of Staphylococcus sp.-like and Salmonella sp.-like bacteria in LM and LF, but no such bacteria was detected in LA. Therefore it concluded that aerobic digestion method could be an effective method in reducing pathogen realized hazards in swine manure. But qualitative analysis is required to further assess this conclusion because only limited microorganisms could be isolated under laboratory condition.

This study was conducted to qualitatively evaluate the effect of ATAD on pathogenicity through culture independent method. The 16s rRNA phylogenetic analysis of the LM, LF, LA were performed using reference pathogenic strains (human and animal) for animal manure and antibiotic resistant strains. The richness (Chao), complexity (H′) and community structure were also calculated and drawn for the analysis.

The result for Chao and H′ have shown decreasing trend. The Chao have dropped over 45% from 140 in LM to 58.15 and 45.67 in LF and LA respectively. The H′ value dropped from 3.58 in LM to 3.42 and 3.28 in LF and LA respectively. The phylogenetic evaluation have shown 36% of LM, 57% of LF and 18% of LA sequences being relatives to reference pathogens.

The decrease in Chao and H′ combined with increase in pathogen relatives in LF, with respect to LM, could be a indication that condition in Lagoon fermentator is favouring selection of pathogens, especially for the one belonging to classes that have shown increase its sized in its community structure (eg. γ - proteobacteria). In LA Chao, H′, and pathogen relatives have decreased with respect to LM. This could be an indication that ATAD is favouring growth of non-pathogenic bacteria, especially for the ones belonging to classes that have shown increase its sized in community structure (eg. C.30trida). Therefore stabilizing swine manure through ATAD could drastically reduce pathogenicity compared to the conventional Lagoon fermentation.

Keywords ATAD; Pathogenic microorganisms; Livestock waste stabilization

Ability of Ralstonia solanacearum phylotype II to adapt to simultaneous stress conditions of oligotrophy and temperature in water

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The Ralstonia solanacearum species complex causes the bacterial wilt disease, which affects economically important crops and ornamentals worldwide. The bacterium is considered a quarantine organism in the European Union, the USA, and Canada. The complex is classified into four phylotypes according to molecular characteristics. Among them, the phylotype (ph) II seems to survive in watercourses as a planktonic form until contact with the next host, retaining pathogenicity. However, there is currently scarce information on the effect that prevailing environmental stresses such as temperature and oligotrophy may have when acting simultaneously on the pathogen populations in aquatic habitats. In this work, population dynamics and adaptations by distinct R. solanacearum ph II from cold or warm zones have been monitored during exposure at various environmental temperatures and different oligotrophic conditions in water.

Survival experiments were conducted with a number of microcosms prepared from samples of sterile natural river water and sterile distilled water. These microcosms were separately inoculated at 106 c.f.u. ml-1 with either of two R. solanacearum ph II strains isolated from cold or warm habitats, and incubated at low (4ºC), temperate (14ºC) and warm (24ºC) temperatures under two different levels of oligotrophy, nutrient limitation (river water) and nutrient deprivation (distilled water). Sampling from each microcosm was periodically done to perform viability tests based on metabolic activity of the stressed cells and dying to microscopically determine the number of total and viable R. solanacearum cells; plate counts for culturability; direct staining for R. solanacearum cell shape observations, and pathogenicity assays by stem inoculation of the stressed cells in susceptible host plants in a greenhouse under quarantine conditions.

Population dynamics at 4ºC revealed that R. solanacearum viable populations of the strains isolated from either cold or warm areas responded to coldness by entering a viable-but-non-culturable (VBNC) state, a mechanism evolved by non-sporulating bacteria in adverse environmental conditions. The cold-induced VBNC state for both ph II strains of the pathogen suggested that these strains were not naturally cold-adapted, even if isolated from cold habitats. In the water microcosms, the VBNC state was dependent on water nutrient contents, since the loss of culturability of the populations was significantly more rapid in conditions of nutrient deprivation than of nutrient limitation, that is, in the natural river water samples. With respect to morphology, R. solanacearum VBNC cells kept typical bacillar shape, unlike VBNC cells from many other bacterial models. Population dynamics at 14ºC and 24ºC revealed adaptations to oligotrophy for both R. solanacearum ph II strains consisting of starvation-survival responses, with cells readily culturable in their initial numbers throughout the experimental period, and transitions of starved bacillar cells to coccooids at both temperatures with significantly higher proportions of coccoid forms at 24ºC, and then pointing out to an influence of temperature on adaptations to oligotrophy. Total and viable populations had similar levels at 4ºC, 14ºC and 24ºC. Interestingly, starved cells retained pathogenicity and were highly virulent at 4ºC, 14ºC and 24ºC on susceptible host plants.

Overall, R. solanacearum ph II proved to be able to adapt itself to various unfavourable conditions of temperature and nutrient scarcity in water, including conditions more resembling to those of natural aquatic habitats, without compromising its disease-inducing capacity. Understanding R. solanacearum ph II adaptations to environmental stresses can help to design strategies to manage the dissemination of this devastating pathogen in natural settings.

Keywords phytopathogenic bacterium; oligotrophic water; nutrient deprivation; temperature; survival responses; VBNC induction
Agrobacterium-mediated transformation of the endophytic fungus *Penicillium pinophilum* associated with sugarcane

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The term endophyte was coined some years ago to refer to interior colonization of plants by microorganisms that usually do not cause damage to the hosts and live most of their life inside of the plant tissues without eliciting any pathogenic symptoms. Some endophytes establish active relationship with their host plants and promote benefits to the plant. The potential for practical applications of endophytes has led to studies addressing the fungi’s ability to control both disease and insect infestations, as well as promoting plant growth. *Penicillium pinophilum* is a well known endophytic fungus that forms symbiotic sugar cane association. Sugar cane is one of the most important cultivated crops in Brazil. Currently, sugar cane importance is increasing considerably around the world due to searching for renewable energy sources, highflying the ethanol used as biofuel. Considering its economical and environmental relevance and the microbial benefits we aimed to evaluate the almost non-known interaction fungi-sugar cane. We developed an efficient Agrobacterium-mediated transformation system for *P. pinophilum*, using as a model the green fluorescent protein (GFP) expression. *P. pinophilum* was transformed by *Agrobacterium tumefaciens* following a modified methodology previously described by de Groot et al. (1998). Cultures of *A. tumefaciens* harboring the binary vectors pFAT2 and pFAT-gfp were grown with shaking in YM liquid medium (25 ml) containing the appropriate antibiotics at 28°C for 24 hours. The *A. tumefaciens* cells were then diluted to an OD660nm of 0.15 in liquid induction medium (IM) supplemented with acetosyringone (200 mM) and the bacterial culture was subsequently grown at 28°C for 8 hours. The mixture fungus-bacterium was placed (200 ml) on a nitrocellulose membrane with a 0.45 mm pore size and 45mm diameter. The membranes were subsequently transferred to Petri plates, carrying potato agar medium that contained hygromycin B (100 μL mL-1) and cefotaxime (200 mM), and incubated at 28°C. We found the best transformation efficiency for conidial transformation was using the co-cultivation period of 48 hours. Putative transformant *P. pinophilum* cells became apparent on the selective media after 5 days of incubation. The mitotic stability of the transferred DNA was confirmed by growing ten transformants for six generations in agar media without selective antibiotics. Resistance to hygromycin B was maintained for all mutants. Fluorescence emission was retained by the transformants and also expressed in sugar cane tissues from inoculated plants with GFP-transformed *P. pinophilum*. Epi-fluorescence light microscopy of two *P. pinophilum* transformants that expressed the green fluorescent protein (GFP) revealed intercellular hyphal growth. We have exploited the qualities of GFP as a reporter to study the interactions between *P. pinophilum* and its sugar cane hosts. The stable integration and expression of the introduced gene into the genome of the recipient fungus indicate that the endophytic fungi may become an excellent tool for delivering and expression of agronomical important genes (e.g. disease, insect resistance and promoting growth) to host plants.

**Keywords** Endophytes; Green fluorescent protein (GFP)

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Antibiogram profile of *Escherichia Coli* isolated from the migratory whistling swans in Hakaluki haor of Moulavibazar district, Bangladesh

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A total of 100 *E. coli* isolates were isolated from the migratory birds of Hakaluki haor of Moulavibazar district and were subjected for in-vitro drug sensitivity test. Ten different drugs like Colistin Sulphate (CS), Gentamicin (GNT), Azithromycin (AZM), Levofloxacin (LVX), Ciprofloxacin (CIP), Tetracycline (TC), Amoxicillin (AMX), Metronidazole (MT) were used in this study to detect the drug sensitivity pattern of those isolates. The antibiogram status of each drug was determined by measuring the diameter of the zone of inhibition due to the diffusion of the agent into the medium surrounding the disc. A high of 96%, 89% and 88% *E. coli* isolates showed the sensitivity to CS, LVF and CIP respectively, followed by AZM (79%), GNT (77%), AMX (63%), TC (46%). No isolate was sensitive to MT (0%).

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Keywords: antimicrobial resistance, drug susceptibility, *Escherichia coli*.
Antimicrobial activity of chitosan against *Fusarium oxysporum* f. sp. *tracheiphilum*

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Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *tracheiphilum*, is an important cowpea disease in the Brazilian Northeast. Control of this disease is difficult, since the use of chemical methods causes environmental damage and induction of resistance in pathogens. An alternative for the treatment of Fusarium wilt is the biological control, which may occur by the induction of resistance in the plant through the application of chitosan in soil. Chitosan, a cationic amino polysaccharide, essentially composed of β-1,4 D-glucosamine (GlcNAc) linked to N-acetyl-D-glucosamine residues, is naturally present in the cell wall of certain fungi, and can also be obtained by chitin deacetylation from the exoskeleton of crustaceans, insects and arthropods. In food technology chitosan is readily seen due to its several functional properties and can be used as an antimicrobial agent. The aim of this study was to investigate the antifungal activity, in vitro, of chitosan, from *Cunninghamamella elegans* UCP 542, against three pathogens *Fusarium oxysporum* f. sp. *tracheiphilum*. Chitosan was extracted from *C. elegans* biomass by alkali-acid treatment. Chemical characterization were effected by infrared spectroscopy (Deacetilation degree) and viscosity (Molecular weight). The effectiveness of chitosan isolated from *C. elegans* in inhibiting the growth of *Fusarium oxysporum* f. sp. *tracheiphilum* was evaluated. Chitosan solutions at concentrations ranging from 10.0 to 0.025 mg/mL was prepared in acetic acid 0.5% (v/v), pH adjusted by 5.5. The antifungal activity was assessed by determining the minimum inhibitory and fungicidal concentration using broth dilution method in Sabouraud medium. Chitosan was replaced with sterile distilled water and 0.5% acetic acid in the positive control. Microbial growth was observed in all positive control. Also, the viability of the *F. oxysporum* was confirmed by verifying their growth in Sabouraud agar without adding chitosan. The chitosan showed the degree of deacetylation and the viscosimetric molecular weight respectively of 85% and 2.72 x 10^4 g/mol. Chitosan showed minimum inhibitory concentration and minimum fungicidal concentration for *F. oxysporum* assayed in 0.5 mg/mL and 4.0 mg/mL, respectively. The exact mechanism of the antifungal action of chitosan is still unknown, but different mechanisms have been proposed, which consider its chemical and structural properties. The results obtained in this study demonstrate the antifungal potential of chitosan against phytopathogenic fungi.

Keywords Polymer; phytopathogenic fungi; antifungal property

Application of *Bdellovibrio*-and-like organisms (BALOs) in integrated pest management

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Numerous bacteria, such as the plant pathogenic species of the genera *Pseudomonas* and *Xanthomonas* are known to cause severe crop losses in agriculture. Several chemical pesticides are used against them successfully but by the use of these compounds the environment is substantially polluted, furthermore, many of them can be harmful for humans as well. A safer solution would be the application of *Bdellovibrio*-and-like organisms (BALOs), which can consume a range of Gram negative bacteria, within the frames of integrated pest management. We have isolated four putative (BALOs) from the rhizosphere of tomato plants and a *Bdellovibrio bacteriovorus* strain was purchased from the culture collection DSMZ. The isolates were found to belong to the *Bdellovibrio*-related species *Peredibacter starrii*. The *B. bacteriovorus* strain was shown to be efficient against all strains of the plant pathogenic *Pseudomonas syringae* tested, while the isolates could consume all the available *Xanthomonas campestris* strains. For agricultural applications the *B. bacteriovorus* and the *P. starrii* isolates can be cultivated on *P. putida* and an apathogenic strain of *Pseudomonas syringae* tested, while the isolates could consume all the available *Xanthomonas campestris* strains. For agricultural applications the *B. bacteriovorus* and the *P. starrii* isolates can be cultivated on *P. putida* and an apathogenic strain of *X. campestris*, respectively. All the strains were shown to keep their predatory activity in the presence of moderate amounts of copper sulfate, therefore they are potential candidates for being used in integrated pest management strategies in combination with a reduced amount of copper-containing pesticides. For the sake of a less complicated maintenance attempts are being made for the isolation of host independent strains from the parental isolates.

The work was supported by the project ‘Jedlik Ányos’ (OM-00136/2007).

Keywords *Bdellovibrio*-and-like organisms; integrated pest management

Keywords *Bdellovibrio*-and-like organisms; integrated pest management
Associated microbiota in soil of organic coffee cultivate

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The fungi microbiota was studied in two coffee organic harvesting in organic system, located at South of Minas Gerais State Region (Três Pontas) and Alto Paranaíba Region (Patrocínio). Soil samples were collected in two different positions: (1) 10 cm far away of the trunk, (2) canopy projection, (3) between the planting lines of coffee and (4) out the coffee plantation area, but into the farm limits. After sampling, the samples were conditioned in boxes and conducted to the EPAMIG-CRSM/EcoCentro Phytopathology Laboratory to realize the microbiological analysis. In both regions there was a predominance of the fungi genus Penicillium potential phosphate solubilizer in relation to the others genera. In the Alto Paranaíba there was a higher number of Forming Colony Units (UFC) of this genus in the dry period, probably due to the weather conditions been favorable to xerophilic fungi. In the same region was detected higher level of P in soil. This fact can be due to higher presence of phosforo solubilizer fungi how the Penicillium genus. The South of Minas Gerais Region favored higher diversity of fungi genera, with more occurrence of the fungi of the genera Aspergillus and Fusarium potential mycotoxins producers.

Key words: organic cultivate, fungi microbiota, Coffea arabica L.

Associations between rumen microbes and cattle feed efficiency

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The rumen and its complex microflora play a major role in supplementing energy for the metabolic functions of cattle. It is therefore an active “bio-reactor” producing many unique bio-products, some of which remain uncharacterized, which are fundamental components for a cow’s daily nutrition, production, and maintenance of health. In addition, the ruminal methanogens, a group of microbes, are known to play a significant part in the production of methane gas which contributes about 15% of total atmospheric emissions from farmed ruminants. Although it is recognized that the rumen is a very complex mixture of different microorganisms, it has been a very challenging area of research, as it is estimated that less than 15% of them can be isolated and grown in the laboratory. The understanding of the biological processes of the microbes in the rumen has therefore remained poor and hence the subsequent linkage between the microbial functions with host performance is not well understood. We will report our recent results of understanding of the associations among rumen microbes including bacteria, methanogen, and fermentation metabolites and host feed efficiency traits. For Bacteria, our results showed that particular bacteria and their metabolism in the rumen may contribute to differences in host feed efficiency under low and high energy diet. For methanogens, the methanogenic community in inefficient animals was more diverse than that in efficient ones under low energy diet and the feed efficient associated as well as diet associated methanogens were identified. For both microorganisms, the copy numbers of 16S rRNA genes were not associated with either metabolites or feed efficiency traits, suggesting the structure/diversity of the community is associated to the host functions. Our attempts to link the ruminal microbial ecology and its functions to cattle’s feed efficiency will supply fundamental understanding of the contribution of rumen microbial community to host biology.
Auto-inducing peptides, comC, D, and E, in a ruminal bacterium, \textit{Streptococcus bovis}

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\textbf{Background}

\textit{Streptococcus bovis} often predominates in the rumen when ruminants are fed a diet containing a large amount of readily fermentable carbohydrate, such as starch. Feeding high-carbohydrate diets generally leads to an increase in lactate production in the rumen, which causes a drop in ruminal pH. Since \textit{S. bovis} is relatively acid-tolerant among ruminal bacteria, the proportion of \textit{S. bovis} in ruminal microbiota often increases when ruminal pH is low. In addition, \textit{S. bovis} produces higher percentages of lactate when culture pH is low, thus suggesting that \textit{S. bovis} contributes to the progress of rumen acidosis. Therefore, it is desirable to suppress the overgrowth of \textit{S. bovis}, or the \textit{overproduction of lactate by \textit{S. bovis}}. It has been proposed that quorum sensing is a bacterial intercellular communication mechanism for controlling gene expression in response to environmental stress or population density. Some acylated homoserine lactones and AI-2 like substances have been demonstrated in some rumen bacteria, but no peptide pheromone has been detected yet. To elucidate the regulatory mechanisms of \textit{S. bovis} growth, we investigated the molecular properties and gene expression of the peptide pheromone-signaling system, consisting of a two-component regulatory system (ComDE).

\textbf{Results}

In \textit{S. bovis}, this signaling system is encoded by three genes, \textit{comC}, \textit{comD}, and \textit{comE}, which encode the precursor of a peptide competence factor, histidine kinase, and a response regulator, respectively. These genes were found to be clustered, but \textit{comC} was present on the DNA strand opposite to the strand harboring \textit{comD} and \textit{comE}. Two homologous genes of \textit{comD} and a homologous gene of \textit{comE} were also present in the neighborhood of the \textit{comCDE} cluster. It was demonstrated by RT-PCR and real-time RT-PCR that \textit{comC} and \textit{comD} are cotranscribed and \textit{comC} is transcribed in a monocistronic fashion. Intracellular \textit{comC}-mRNA level increased sharply during the initial exponential growth, and decreased abruptly after the middle exponential phase. Therefore, \textit{comC} transcription appears to change with the growth stage.

The growth rate on glucose was decreased by disrupting \textit{comCD}, indicating that the peptide pheromone-signaling system affects the growth of \textit{S. bovis}. The ratio of formate to lactate produced in 1 h during the late log phase was not different significantly between the \textit{comCD}-disrupted strain and the parent strain, \textit{JB1}. These results suggest that \textit{ComC} affects growth without affecting fermentation pattern. In a \textit{comC}-disrupted mutant, the transcript level of \textit{comE} was decreased, and the expression of several functional proteins was changed. However, addition of \textit{comC} peptide to the cultures of \textit{JB1} increased growth rate and transformation efficiency. These results suggest that \textit{S. bovis} ComC stimulates growth and enhances competence.

\textbf{Conclusions}

Deletion of \textit{comC} and \textit{comD} decreased growth rate, which may be related to the change in the expression of several functional proteins. The growth rate and transformation efficiency were increased by adding mature \textit{ComC} peptide. Thus, the peptide pheromone-signaling system might be involved in the regulation of growth and the enhancement of competence in \textit{S. bovis}. These results may contribute to the future development in the control of \textit{S. bovis} overgrowth and the prevention of ruminal acidosis.

\textbf{Acknowledgments}

This study was supported in part by a Grant-in-Aid for Scientific Research (No. 20780196) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (MEXT).

\textbf{Keywords} peptide pheromone; rumen bacteria; ruminal acidosis; \textit{Streptococcus bovis}; two-component signal transduction system

**Auxin-induced recovery of phytoplasma-infected periwinkle**

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Phytoplasmas (genus ‘Candidatus Phytoplasma’) are endocellular plant pathogenic bacteria that cause numerous economically important diseases. Symptoms like leaf yellowing, shortening of internodes and stunting, extreme proliferation of the shoots and formation of witches’-brooms, phylody and virescence are often associated with the infections. In many cases phytoplasmas cause death of their plant hosts. Many research efforts were made in order to find a suitable method to fight against phytoplasma diseases. Phytoplasmas have reduced genomes of 530 – 1130 kb and are highly dependent on the intake of the nutrients from their hosts. Infections caused by phytoplasmas lead to developmental disorders in infected hosts via deregulation of developmentally important genes while the presence of these pathogens disturbs the normal transport through the phloem causing numerous physiological and biochemical changes in infected hosts including the changes in plant growth regulators balance. This fact was the basis for the idea to try to eliminate phytoplasmas by treating infected plants with auxins.

In vitro grown Catharanthus roseus shoots infected with 3 different ‘Candidatus Phytoplasma’ species were treated with two auxins. Tested plant growth regulators, indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA), induced remission of symptoms (recovery) in all phytoplasma-infected plants. The time period and concentration of the auxin needed to induce recovery was dependent on the ‘Candidatus Phytoplasma’ species and the type of auxin. IBA-treatment eliminated ‘Ca. P. asteris’ (strain HYDB) from the host tissue while ‘Ca. P. solani’ (strain SA-1) and ‘Ca. P. ulmi’ (strain EW-Y) persisted in the host tissue despite the obvious recovery of infected plant. To elucidate the possible mechanism of host recovery and ‘Ca. P. asteris’ elimination from \textit{C. roseus} shoots caused by indole-3-butyric acid (IBA)-treatment, \textit{H}_{2}\text{O}_{2} and related enzymes, stem anatomy and callose deposition in phloem tissue, endogenous auxin levels and general methylation levels were measured and compared for non-infected periwinkles, phytoplasms infected with different ‘Candidatus Phytoplasma’ species and phytoplasma-recovered periwinkles. Differences in methylation of the host plant genome after the treatment with auxin revealed that epigenetic changes induced in periwinkle by IBA treatment might be responsible for the elimination of ‘Ca. P. asteris’ from the infected plant. In nature, rare cases of spontaneously recovered plant species are known. However, the molecular mechanism of phytoplasma recovered periwinkles upon the treatment with IBA is different than the proposed recovery mechanism in spontaneously recovered plants where peroxide and related enzymes are the proposed cause of the recovery.

Our results show that in the case of some ‘Candidatus Phytoplasma’ species, auxin-treatment could be used to eliminate phytoplasmas from plants.

\textbf{Keywords:} bacteria, elimination
Bacteria in salt-marsh sediments: Influence of plant colonization on abundance, physiological diversity and heterotrophic activity (Ria de Aveiro, Portugal)

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The diversity and activity of microorganisms are strongly influenced by plant colonization. Microbial communities associated with plant rhizospheres are highly diverse and often more active than the microflora of bulk sediments. The aim of this study was to characterize and compare the microbial communities associated from two types of salt marsh sediments colonized either by Spartina maritima or by Halimione portulacoides, two halophyte plants often proposed for phytoremediation approaches in impacted estuaries. Sediments cores of 16 cm length were collected from a non-vegetated area (control) and from each of monospecific vegetation banks. Porosity, sediment grain-size and nutrient concentration were measured along vertical profiles with a 2cm pace. Microbiological descriptors were assessed at the same sediment horizon. Potential rates of ectoenzymatic activity (β-glucosidase, aminopeptidase, α-glucosidase, arylsulfatase and phosphatase) assessed by the hydrolysis of fluorogenic model substrates, showed generally higher activity in the vegetation banks in relation to control sediments that were also vertically more homogeneous. Aminopeptidase showed the highest activity rates (0.1992.7 mmol gdw⁻¹ h⁻¹), and α-glucosidase presented the lowest (0.043.2 mmol gdw⁻¹ h⁻¹). The physiological diversity of bacterio-,rhobenthos assemblages, characterized by the profiles of utilization of sole-carbon-source (Biolog Ecoplates) showed that only the communities from the upper sediment layer of the S. maritima and the H. portulacoides banks exhibit consistent differences in terms of physiological profiles. Total prokaryote abundance (5.90x10⁻² – 2.71x10⁻¹² cells gdw⁻¹) was higher in surface sediments. The relative abundance of the domain Bacteria was approximately 40% of total cell counts with the highest proportion occurring in the surface layer. The Archaeal domain contained approximately 25% of total prokaryote cells with a homogeneous vertical distribution. The relative abundance of sulfate-reducing bacteria (SRB) was approximately 3% of total cell counts in control sediments and at the H. portulacoides bank and 7% at S. maritima. The analysis of 16s rDNA PCR-DGGE profiles suggest a high diversity of the bacterial communities in the rhizosphere of the two salt marsh plants. The different patterns obtained, indicate that the communities are structurally distinct at the two vegetation banks. However, at the S. maritima bank there is also considerable spatial variability in the structural diversity of the sediment bacterial communities. The analysis of the abundance, diversity and structure of microbial communities of salt marsh plant rhizospheres suggest the existence of specific interactions between roots of estuarine plants and sediment microbial communities. *Archaea* represent an important fraction of the prokaryote assemblages regardless of the chemical environment of each sediment depth layer. Changes in plant colonization of intertidal sediments banks affect the metabolic processes of organic matter recycling in estuarine systems and the structure and physiological profile of the bacterial communities involved.

**Keywords:** Rhizosphere; Salt marsh; Extracellular enzymatic activity; Sole-carbon source utilization profiles; Bacterial and Archaea distribution; SRB; DGGE profiles

Bacterial ACC-deaminase induced changes in root architecture and their influence on nodulation, growth and yield of *Cicer arietinum* L.

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Bacteria containing 1-aminocyclopropane-1-carboxylate (ACC)-deaminase in the vicinity of roots may influence plant growth by modifying root architecture through their potential to regulate ethylene (C₂H₄) synthesis in plant roots. The present study illustrates changes in root growth and development in response to inoculation with rhizobacteria containing ACC-deaminase and their resultant effects on nodulation, growth and yield of chickpea (*C. arietinum* L.) under controlled (axenic) and natural conditions. Approximately 138 isolates capable of utilizing ACC as the sole source of N were isolated from rhizosphere of chickpea plants and evaluated for their growth promoting effects on chickpea seedlings under controlled conditions. Four strains were the most efficient in improving root growth of chickpea seedlings as compared to uninoculated control under axenic conditions (jar trial). Among these strains, *Serratia proteamaculans* (J119) was the most effective plant growth promoting rhizobacterium associated with plant rhizospheres that was also vertically more homogeneous. Aminopeptidase showed the highest activity rates (0.1992.7 mmol gdw⁻¹ h⁻¹). The physiological diversity of bacterio-,rhobenthos assemblages, characterized by the profiles of utilization of sole-carbon-source (Biolog Ecoplates) showed that only the communities from the upper sediment layer of the S. maritima and the H. portulacoides banks exhibit consistent differences in terms of physiological profiles. Total prokaryote abundance (5.90x10⁻² – 2.71x10⁻¹² cells gdw⁻¹) was higher in surface sediments. The relative abundance of the domain Bacteria was approximately 40% of total cell counts with the highest proportion occurring in the surface layer. The Archaeal domain contained approximately 25% of total prokaryote cells with a homogeneous vertical distribution. The relative abundance of sulfate-reducing bacteria (SRB) was approximately 3% of total cell counts in control sediments and at the H. portulacoides bank and 7% at S. maritima. The analysis of 16s rDNA PCR-DGGE profiles suggest a high diversity of the bacterial communities in the rhizosphere of the two salt marsh plants. The different patterns obtained, indicate that the communities are structurally distinct at the two vegetation banks. However, at the S. maritima bank there is also considerable spatial variability in the structural diversity of the sediment bacterial communities.

The analysis of the abundance, diversity and structure of microbial communities of salt marsh plant rhizospheres suggest the existence of specific interactions between roots of estuarine plants and sediment microbial communities. *Archaea* represent an important fraction of the prokaryote assemblages regardless of the chemical environment of each sediment depth layer. Changes in plant colonization of intertidal sediments banks affect the metabolic processes of organic matter recycling in estuarine systems and the structure and physiological profile of the bacterial communities involved.

**Keywords:** ACC-deaminase; rhizobacteria; nodulation; root growth; legumes
Bacterial community structure in the rhizosphere of three cactus species growing in xerophytic highlands in central Mexico

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INTRODUCTION. The Tehuacan-Cuicatlan reserve, in the central highlands of Mexico, is an arid area of unique plant biodiversity constituted mainly by xerophytes, with exceptional concentrations of rare and endemic species. From the 75 registered species of Cactaceae in this area, 21 are endemic. This natural diversity is being threatened by several human activities; however, the uncontrolled recollection of plants is probably the main threat to cactus species. Management and propagation of cactus plants normally do not take into account the microbial community associated to their roots, which probably are related to plant important processes (i.e. nutrient solubilisation, N2 fixation). Based on their growth type 3 cactus species were chosen; an herbaceous Mammillaria carnea (Mc), a shrub Opuntia pilifera (Op) and a species with tree-like growth; Stenocereus stellatus (Ss).

The objective of this work was to assess by culture- and non culture-based methods, the diversity and structure of the bacterial communities present in the rhizosphere of the 3 cactus species; and non-rhizospheric soil, focusing on changes related to season (rain - dry) and plant species.

METHODS. Rhizosphere samples of the 3 cactus species (Mc, Ss, Op) and non-rhizosphere soil (nr) were taken during dry and rainy season. Samples were used for viable counting and to isolate bacteria in; complex medium (TY) and Nitrogen-free medium (Nf). Isolates were first selected by colony morphology and further characterized with a polyphasic approach. Samples were also analyzed by molecular methods based on DNA extraction, PCR amplification, DGGE and SARST profiling, and 16S rDNA sequence analysis.

RESULTS. Viable counts were performed on both media and during dry and rainy season. ANOVA demonstrated that: a) main differences in abundance of cultivable bacteria were related to season, being the CFU values higher during the rainy period. b) No CFU differences were found among the 3 rhizospheric samples, in both; media and season. c) The nr samples reported lower CFU values than the rhizospheric samples in both media, but only for rainy season. A total of 40 isolates were obtained in TY medium, and 39 in Nf medium, selection was based on colony morphology. These morphotypes were grouped by means of V6-V8 16S rDNA, PCR followed by DGGE, allowing reduction to 21 ribotypes for TY, and 19 for Nf. All the isolates were placed in 5 taxonomic groups: α-, β-, and γ-Proteobacteria, Actinobacteria and Firmicutes, the majority of the isolates belonged to the α- (41%), γ- (19%) Proteobacteria and Actinobacteria (20%). Ochrobactrum was the most represented genus with 33% of the isolates, found in rhizospheric as well as nr soil. A number of the genera also showed seasonal differences.

DGGE bacterial community profiling was performed by a computer-aided banding-pattern analysis. Under this approach, rhizospheric and nr profiles of the dry season clustered together, while rhizospheric profiles of the rainy season formed a distinct group. SARST profiling was applied only to 2 samples from rainy season; nr and Mc. The later sample probe to be more diverse as representatives of 11 bacterial were found. Interestingly, with this technique the most represented taxa was Actinobacteria with 44% of the retrieved sequences, while the Proteobacteria that was the most abundant taxa for cultivables, was the third represented group with 16% of the sequences.

Keywords cactus rhizosphere, DGGE, SARST, Mammillaria, Stenocereus, Opuntia

Bacterial community structure of no phosphate input management agroecosystems

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Soil erosion and eutrophication of water courses are caused by accumulation and runoff of highly supplemented mineral macronutrients (fertilisers), like nitrogen (N) and phosphorous (P) in agricultural soils which are used to increase crop yield. Sustainable mineral management is necessary for non-renewable fertilisation sources like P, to meet future demands and to overcome the negative impacts on the environment. Phosphate solubilising microorganisms are an important factor promoting P availability for plant growth with the potential to solubilise the adsorbed mineral nutrients and make this available to the plant in soil. The affect of management practices on the structure of these microbial communities is still in its infancy also not much is known regarding the affect of no P inputs on overall community structure in soil. In this study we set out to analyse the affect of management practices on microbial community structure by sampling and analysis of experimental plots which are managed with conventional and no input P source for last 13 years. The Knockbeg field plots are located in Carlow, Ireland managed by Teagasc. These plots are grown with continuous monocultures of wheat and barley crops. The overall structure of bacterial community was analysed from these plots by using molecular tool like PCR-DGGE and 16S rRNA gene library analysis.

The results based on PCR-DGGE and 16S rRNA gene library analysis from different field sites receiving conventional and no P inputs suggest a variable microbial community structure within no P inputs compared with the conventional input regimes. The structural profile generated for different sampling periods and input regime using PCR-DGGE shows a greater seasonal affect compared to no P input regimes. However, the exact functional diversity of the microorganisms present in soil will require further analysis, for example analysing functional genes for phosphate solubilisation in soil and by soil metagenomic library analysis.

Keywords: Runoff, Eutrophication, 16S rRNA, PCR-DGGE.

Acknowledgement

This work is funded by the Department of Agriculture and Food stimulus 2 programmes, Ireland.

References:
Bacterial diversity in the nodules of soybean.

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Soybean (Glycine max L. Merr.) can form root nodules and establish symbiosis with Bradyrhizobium. The nitrogen fixing association between nodule-forming bacteria and host legume plants has been well characterized. On the other hand, occurrence of non-symbiotic bacteria inside the root nodules and its interaction with host plants has been poorly documented. To analyze the diversity of non-symbiotic endophytic bacteria in root nodules, we isolated and characterized several bacterial strains from nodules of soybean. Root nodules were collected from soybean plants (cv. Entrei) grown in the field during three growth phases: flowering, pod development and seed development. A fast-growing bacterium on the YMA medium from surface-sterilized nodules was isolated as a non-symbiotic endophytic bacterium. The percentage of nodules coexisted symbiotic Bradyrhizobium strains and non-symbiotic bacteria was 9 and 2% in the flowering and pod development phases, respectively. The frequency of the isolation of non-symbiotic endophytes from nodules increased to 55% in the seed development phase. We sequenced a fragment of 16S rDNA gene of several non-symbiotic isolates from nodules and found that the isolates were close to the genera Agrobacterium, Rhizobium and Enterobacter.

Keywords: diversity; nodule; non-symbiotic bacteria; soybean

Acknowledgements: This work was supported by the Research and development projects for application in promoting new policy of Agriculture Forestry and Fisheries; the Ministry of Agriculture, Forestry and Fisheries, Japan.

Bio-Organic Fertilizers an Essential Alternative to Harmful Chemical Sources

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The use of chemical fertilizers is already documented as having a harmful affect on the environment. Almost all planters and growers know that using chemical fertilizers for the long term will damage the soil and turn the lands into unproductive plots. The health affects of using chemical fertilizers on the surrounding flora and fauna has already been established to be very negative, yet we still see farms continuing to use chemical fertilizers. Application of the large amounts of chemical fertilizers will lead to decrease the water quality around the farms substantially and drop the agricultural products every year. In this situation, the farmers have to use increasing amounts of fertilizers just to keep their lands productive. Organic fertilizers are more cost effective when used over the medium to long-run compared to chemical fertilizers. Organic fertilizers have no such negative affect on the environment. They promote healthy bacteria and enzymes growth in the soil which nourishes the soil and thus any plant that uses the soil. Organic fertilizers pose no threat to the local water system as very little if any leaches away to the surrounding water. The soil and its microbes will consume the majority of the organic fertilizer when it is applied. Organic fertilizers are not poisonous and thus have no affect on the balance of local flora and fauna. There are no poisons in organic fertilizers thus there do not need any handling precautions to be used safely. Biofertilizers will provide any farmer with significant benefits to the plant harvest quality and also quantity without any worry about health or environmental impact or complications. Long term use of Biofertilizer will bring balance back to soils that have already been spoiled by access use of chemical fertilizers. By using Bio-Organic Fertilizers it does not need to be worry about many soil and health disorders as well as environmental complications or any other adverse reactions from the use of harmful chemical fertilizers. This paper is going to review the benefits of using bio-organic fertilizers for agricultural food production, in terms of soil, water as well as environmental sources compared to increasingly use of chemical fertilizers in many parts of the world.

Keywords: organic fertilizer, biofertilizer, chemifertilizer, benefits, environment.
Biocatalysts of root wilt of chickpea by *Rhizobium* and Azotobacter in *Fusarium* infested soil

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A pot experiment was designed to study the effect of *Rhizobium* and Azotobacter, in alone and in combination, as biocontrol against *Fusarium oxysporum* which cause root wilt in chickpea. A recommended dose of fertilizer (30-60 kg NP ha\(^{-1}\)) was applied to all the treatments. Chickpea (var. AUG 242) was seed coated by inoculums and fungicide. Ten seeds were sown in each pot containing soil, infested by *Fusarium oxysporum*. Germination percentage was recorded one week after sowing. *Fusarium oxysporum* delayed as well as suppressed the germination in control. Co-inoculation of *Rhizobium* and Azotobacter showed highest germination (97.3%) Germination was 80% and 65% in *Rhizobium* and Azotobacter inoculated seeds respectively, compared to control (17.5%). Fungicide treated seeds show 75% germination. Two week after the sowing germination became >90% in all the inoculated treatments but it remain 85% in control. Disease was initiated forty days after the germination in all the treatments. Mortality percentage was recorded at three stages i.e. germination, flowering and pod formation. At germination maximum mortality was in control (16.6%) while it was 3.35% in both co-inoculated and fungicide treated plants followed by 6.65 and 7.5% in plants inoculated by *Rhizobium* and Azotobacter respectively. At flowering co-inoculation and *Rhizobium* alone showed 8.35% compared with control (25%). Mortality was 19% in Azotobacter inoculated plants. At pod formation control was fully wilt while other treatments showed no more wilting. Root parameters were recorded at flowering. Results showed that co-inoculation of *Rhizobium* and Azotobacter produced more no. of nodules (48 pl \(^{-1}\)), higher nodular mass (0.41g pl \(^{-1}\)), more root length (35.75cm),shoot length (35.62cm) root weight(19.25g pot \(^{-1}\)) and shoot weight (29.25g pot \(^{-1}\)) than control having 26 pl \(^{-1}\) of nodule,0.19g pl \(^{-1}\) nodular mass,30cm and 27.5cm root and shoot length respectively while15.75g pot \(^{-1}\)root weight ant 23g pot \(^{-1}\) shoot weight was recorded. In single treatments weight and no. of nodules (0.36g pl \(^{-1}\) and 46, respectively) were recorded with *Rhizobium* followed by Azotobacter 39 nodule pl \(^{-1}\) with 0.32g pl \(^{-1}\) nodular mass. In co inoculation dry biomass was significantly higher (33.67 g) compared with control (17.37g). *Rhizobium* alone produced biomass 30.60g which is statistically at par with fungicide treatment. Azotobacter produced significantly higher biomass 28.83g pot \(^{-1}\).It is concluded that co-inoculation of *Rhizobium* and Azotobacter give better control against the soil borne pathogen to a significant extent.

**Key words:** *Fusarium oxysporum*, *Rhizobium*, Azotobacter, chickpea

Biological activity in a soil amended with pulp mill sludge: A field study

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The biological wastewater treatment plants produce large quantities of sludge which require disposal. The controlled disposal of sludge in soils is an alternative that requires its characterization and pre-treatment for use in a continuous and environmentally safe way. The utilization of sludge in agricultural field or degraded soil is gaining popularity as a means of waste disposal and as alternative to soil mineral fertilizer. The sludge generally contains high organic matter content, microorganisms, macronutrients (P, N, and K), microelements (Zn, Cu and Fe) and inorganic substances (silt, clay and calcium carbonate). The sludge application to the soil can promote the improvement of soil structure, the adjustment of pH, and the addition of plant nutrient and therefore increase productivity. In Chile great amounts of sludge from pulp and paper mill wastewater treatment plants are generated annually, and most of them are placed in landfill. This sludge generally contains organic substances such as cellulose, lignin and microorganisms; as well as, inorganic substances and low content of heavy metals. Today the sludge application in acidic soils is an attractive alternative as improved of degraded soils.

The objective of this study was to evaluate the long-term effect of different levels of pulp mill sludge on biological parameters of an Andisol. The soil used in this study was an Andisol belonging to Freire series (pH, 5.4; OM, 10%; P, 17.5 mg kg\(^{-1}\)); N, 19.2 mg kg\(^{-1}\)). The sludge used was a secondary sludge obtained from the bleached kraft mill wastewater treatment plant (aerated pond), and was collected from a landfill after one year disposal (pH, 7.0; OM, 75%; N, 566 mg kg\(^{-1}\); P, 313 mg kg\(^{-1}\)). The experiment was conducted at the Maquehue experimental station located in Freire, Chile. The experimental design was set up as randomized blocks with three replicates. Each plot measured 6 m x 2 m. The sludge application in the soils was 0, 10, 20 and 30 t ha\(^{-1}\), divided in four applications during one year. Periodically, the biological characteristics such as CO\(_2\) evolution, fluorescein diacetate hydrolysis (FDA) and acid phosphatase activity, at 20, 40 and 60 cm of soil depth, were evaluated.

After 30 days of the first sludge application, an increment of soil respiration at 20 cm depth with the sludge addition was observed, due to the activity of soil microorganisms was stimulated by the presence of fresh organic matter from the sludge. The soil respiration was lower at 40 and 60 cm depth, but no influence of sludge application was observed. Different situation was observed for FDA and acid phosphatase activity. A decrease in activity with the sludge application at rates of 10 and 20 t ha\(^{-1}\) was observed at the first 20 cm depth of soil, while recovery tendency was observed with the rate of 30 t ha\(^{-1}\). The increase of phosphatase activity in sludge amended soil at rate of 30 t ha\(^{-1}\) could be due to the high phosphorus level delivered from the sludge. The enzymatic activities diminished with the increase of soil depth. However, this tendency was notary in un-amended soil.

This work shows preliminary results obtained recently as parts of a long study in field condition. We can conclude that microbial activity of soil is modified with the sludge application, increasing respiration and phosphatase activity, mainly with the high level of the sludge application. Leachates studies of macro and micro elements through the soil depth and the repeated sludge application could help to explain the real effect of sludge application on the soil microorganisms.

**Keywords:** acidic soil, pulp mill sludge, soil respiration, phosphatase, FDA

**Acknowledgements:** This study was financed by the project FONDECYT 1080427.
Caracterization of soil suppresion to Fusarium wilt

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Soil suppression is a fact for many plant diseases in different areas of the world, particularly those soils that are naturally suppressive to diseases induced by *Fusarium oxysporum*. The purpose of this research was to study the suppresiveness characteristics to Fusarium “wilt” of two different soils from the Northwestern of Cádiz (Spain).

Two models were designed: *Fusarium oxysporum* f. sp. niveum-watermelon and *Fusarium oxysporum* f. sp. melonis-melon, that were artificially inoculated in two commercial soils used for testing the suppresiveness.

The microbial populations in soils could be responsible for suppressiveness. Nearly 50 different microorganism isolates, including bacteria, actinomycetes, and fungi, were collected from soils and “in vitro” antagonism for all of them was studied later.

Nonpathogenic isolates of *Fusarium oxysporum*, *Fusarium solani*, *Trichoderma* spp, *Penicillium* spp, and other several microorganisms isolated from soils showed different levels for “in vitro” suppressiveness when they were evaluated against two pathogenic isolates of *F. oxysporum* f. sp. niveum and *F. oxysporum* f. sp. melonis.

We conclude that there is a microbiological suppresiveness in these soils, it is based on microbial interactions between the pathogen and populations of the saprophytic microbiota, that are higher in one of the soils.

**Keywords:** *Fusarium*, suppresiveness, biological control, suppressive soil

Characterisation of soil fungal communities in healthy forest stands and in infected with Heterobasidion parviporum and Armillaria spp.

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Factors by which soils can be suppressive to different pathogens can involve biotic (soil microflora) and/or abiotic elements (soil physicochemical properties), and they may be different with various pathogens. There are suggestions that the main agents in soil suppressiveness are microbial.

The study was undertaken to characterize soil fungal communities of forest stands infected with root rot fungus *Heterobasidion parviporum* and honey fungus *Armillaria* spp. The objectives of the study were to characterize and compare soil fungal communities using following methods: estimation of the amount of cultivable microorganisms colony forming units (CFU) - determination of typical fungal genera followed by calculations of Shannon – Weaver diversity index; extraction of total soil DNA; PCR amplification of the fungal nuclear ribosomal fragments of the obtained DNA followed by ARDRA and calculations of Shannon – Weaver diversity index; and quantitative PCR with universal fungal primers and *Trichoderma* spp. specific primers.

Two 40 years old spruce (*Picea abies* (L.) Karst) stands in *Oxalidosa* and *Myrtillus-polytrichosa* forests, infected with *H. parviporum* were analyzed on Sud-podzolic soils (Cutanic, Stagnic Albeluvisols) and Illuvial humus podzol (Placic, Rustic, Albic, Stagic Podzole) according to Latvian soil classification and FAO WRB soil classification. In each stand one sampling plot was established.

Infection of the analyzed forest stands by parasitic fungi is reflected by significant decrease of the number of CFU of filamentous fungi and by slight decrease of cultivable microorganisms CFU and fungal diversity (with exception of Shannon – Weaver diversity index of cultivable filamentous fungi of sampling plots infected with *Armillaria* spp.). In *Armillaria* comparison with data about healthy forest stands from our previous publication. Soils in sampling plots infected with *Armillaria* spp. have decreased proportion of fungal DNA from the total soil DNA but increased proportion of *Trichoderma* spp. DNA from fungal DNA.

**Keywords:** Fungal diversity, Shannon – Weaver diversity index, ARDRA, real time PCR, *Heterobasidion annosum*, *Armillaria*, *Trichoderma*. 
Characterization of plant growth promoting traits of Methylobacterium oryzae CBMB20 isolated from rice and its effect in efficient use of organic manure for higher yield and growth promotion of red pepper

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A pig-pigmented, facultative methylotrophic bacterial strain Methylobacterium oryzae CBMB20 was isolated from stem tissues of rice. CBMB20 was able to utilize 1-aminocyclopropane-1-carboxylate (ACC) as a nitrogen source and produced ACC deaminase. Strain CBMB20 is also able to produce indole acetic acid production and showing acetylene reductase activity. First we have checked the plant growth promoting potential of CBMB20 under gnotobiotic conditions using pouch assay. Inoculation of CBMB20 was able to enhance total dry weight by 21.7%. For further detailed study a greenhouse experiment was conducted to examine the effects of inoculation of alone CBMB20 and coinoculation with arbuscular mycorrhizal (AM) fungi on the growth of red pepper (Capsicum annum L.). Inoculation of pepper plants with the CBMB20 resulted in a significant increase in root length and root fresh weight compared to untreated control plants. The combined inoculation of CBMB20 and AM fungi significantly increased various plant growth parameters and chlorophyll content compared to uninoculated controls. In addition, the combined inoculation of CBMB20 strain and AM fungi resulted in significantly higher nutrient accumulation in the roots and shoots of red pepper plants compared to uninoculated controls. The micromituent content of the red pepper plants also increased in most of the inoculation treatments. In the present study we are trying to understand the relationship between PGPR belonging to the genus Methylobacterium and in consortium with AM fungi. The effects of either single or combined inoculation with Methylobacterium strains and AM fungi on growth and macro- microscopic shoot was evaluated in Capsicum annum L. plants in greenhouse experiments. The establishment of the microorganisms and their effect on the population of other bacteria was also investigated. Since red peppers require a greater amount of important but scarce nutrients, inoculation with favourably interacting microbes can provide an alternative to chemical fertilizers. Further research on in-depth understanding of the co-operative microbial interactions will facilitate the successful application of microbe-based products in biotechnology. For studying the colonization of CBMB20 in the rhizosphere and phyllosphere of red pepper plants, CBMB20 was tagged with gfp gene through biparental mating method. The plasmid pJ1820/E. coli S17-1 was used for gfp tagging. The presence of gfp in the purified transformants was confirmed by PCR amplification using the specific primers YL065 (F) 5’-GGGATCTTATAGGGGCAAAA-3’ and YL066 (R) 5’-TCTATGCTATGTGAATCCT-3’. The transformability stability was determined by restreaking a single colony from selective medium onto tryptic soy agar without any selection pressure. After completing 12 rounds of restreaking at 5- day intervals, the colonies were scored for kanamycin resistance and presence of gfp through PCR analysis. Furthermore, fresh colonies scraped off from agar plates after washing in sterile distilled water were starved at 4°C for 3 weeks, and the cell suspensions were then plated onto AMS succinate media with and without kanamycin. The colony, cell morphologies, and growth of the gfp derivative in TSA, AMS succinate, and AMS with 0.5% (v/v) methanol were compared with that of wild type. In the present study we are presenting the effect of organic fertilizer on the plant growth of red pepper in presence and absence of gfp tagged Methylobacterium oryzae CBMB20 as soil or foliar application greenhouse condition.

**Keywords**: methylbacteria, AM fungi, co-inoculation, soil application, foliar application, greenhouse, gnotobiotic

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Characterization of rhizospheric bacteria isolated from maca (Lepidium meyenii W.) in the highlands of Junin-Peru

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Lepidium meyenii Walpers, also known as maca, is the only member of the Brassicaceae family cultivated in the Central Andes of Peru, in the ‘Bombon’ highlands between the departments of Pasco and Junin at 3800 and 4500 m.a.s.l., where it has been found the most genetic variability. This crop develops in very cold ecosystems with temperatures between 3 and 7°C during day until 10°C at night. There is high sun light, frequently freezing conditions, strong winds, and acid soils (pH = 5) [1]. On the other hand, there is a number of rhizospheric microorganism with many beneficial capabilities on the roots and foliage that can be a direct (nitrogen fixing, phytohormones production, siderophores and phosphate solubilization) or indirect (fungi inhibition, systemic acquired resistance and other) way.

The aim of this study was characterize the bacteria diversity from maca for their future application as inoculants in the stressing conditions of maca breeding. Roots in different phenology stages were collected. Pseudomonas, free-life diazotrophic bacteria (FLD), actinomycetes and Bacillus sp. were isolated in selective mediums under mesophilic conditions (28°C). For characterization of PGPR capability of the isolates, there was evaluated IAA (Indole-3-acetic acid) production and phosphate solubilization at 5, 14 and 28°C. Furthermore, the effect of the strains in the promotion of maca seeds germination was made under dark conditions and temperatures between 15 and 18°C during 3 days. Finally, the strains were also finger-printed using BOX-PCR and sequenced with rD1 and rD2 16S rDNA primers.

355 bacteria were obtained, 108 FLD, 29 actinomycetes, 109 Pseudomonas sp. and 109 Bacillus sp. At 28°C, high levels of IAA production and phosphate solubilization were obtained in 50% of FLD and Pseudomonas sp. such 40A, 42A, 14A strains. The group of FLD bacteria had a better solubilization of the Bicalcic phosphate whereas the group of Pseudomonas sp did better in tricalcic phosphate. In contrast, Bacillus sp and actinomycetes showed lower levels in these two phosphate sources. About the tests realized at low temperatures, the 56% of the strains showed a high level of IAA production and a good phosphate solubilization at 1°C in 5A, 40A and 42. The 5A strain showed the same solubilization at 5°C and 14°C. Also an improvement in IAA production was found at 14°C in Pseudomonas sp. strains (Table 1).

<table>
<thead>
<tr>
<th>Strain</th>
<th>IAA (μg/ml)</th>
<th>Bi-Ca* (cm)</th>
<th>Tri-Ca* (cm)</th>
<th>IAA (μg/ml)</th>
<th>Bi-Ca* (cm)</th>
<th>Tri-Ca* (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free-life diazotrophs (FLD)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5A</td>
<td>1.97</td>
<td>0.4</td>
<td>17.66</td>
<td>0.35</td>
<td>15.37</td>
<td>0.6</td>
</tr>
<tr>
<td>5A</td>
<td>2.6</td>
<td>0.6</td>
<td>19.34</td>
<td>0.6</td>
<td>19.57</td>
<td>0.5</td>
</tr>
<tr>
<td>Pseudomonas sp</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pd45</td>
<td>1.87</td>
<td>0.5</td>
<td>19.82</td>
<td>0.6</td>
<td>5.89</td>
<td>0.1</td>
</tr>
<tr>
<td>B15</td>
<td>1.87</td>
<td>0.7</td>
<td>19.32</td>
<td>0.6</td>
<td>5.99</td>
<td>0.1</td>
</tr>
<tr>
<td>Bacillus sp.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B1</td>
<td>0</td>
<td>0</td>
<td>10.13</td>
<td>0</td>
<td>4.59</td>
<td>0</td>
</tr>
<tr>
<td>B2</td>
<td>0</td>
<td>0</td>
<td>13.04</td>
<td>0.9</td>
<td>29.97</td>
<td>0</td>
</tr>
</tbody>
</table>

**IAA evaluated at 2 days. Phosphate solubilization evaluated at 11 days**

Then, 10 of the strains (3 FLD, 2 Pseudomonas sp, 3 Bacillus sp and 1 actinomycete) were selected in order to investigate the effect on maca seeds germination. B2, Pd45, 12act, 5A and Pd42 strains improved the germination percentage in contrast to the non-inoculated control. With the BOX-PCR analysis, a great genetic variability was found in each studied bacterial group. The PGPR potential showed in the isolated strains turns to be a promise in the agriculture in order to raise the yield and nutrients content in the maca roots. Acknowledgements: Grant FONDECYT – CONICYTE, FONDECYT 111-09, Ecodina SAC.

**Keywords**: PGPR bacteria, IAA, phosphate solubilization, germination, psicrophilic bacteria.
Cicer arietinum growth promotion by Ochrobactrum intermedium and Bacillus cereus

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Aims: This study assessed the plant growth-promoting ability of the bacterial strains Ochrobactrum intermedium CrT-1 and Bacillus cereus S-6.

Methods and Results: Two chromium resistant bacterial strains isolated from chromium-contaminated wastewater and soils were identified as Ochrobactrum intermedium CrT-1 and Bacillus cereus S-6. These strains were inoculated on seeds of chickpea (Cicer arietinum var NM-88), which were germinated and grown under chromate salts (300 μg ml⁻¹ of CrCl₃ or K₂CrO₄). The data show that Cr(VI) was more toxic due to its better availability to plants roots as compared to Cr(III). The major part of Cr(VI) supplied to the seedlings was reduced to Cr(III) in the rhizosphere by the bacterial strains thus lowering the toxicity of chromium to seedlings.

Conclusions: Strains have significant Cr(VI) resistance and reduction potential and have ability to enhance chickpea plant growth under chromium stress.

Significance and impact of the study: These strains could be utilized for the growth of economically important cash crops as well as for the bioremediation of chromium polluted soils.

Keywords: Bacillus cereus, Chromium, Ochrobactrum intermedium, Cicer arietinum, Cr(VI) reduction, Heavy metals
Colonization and migration abilities of Erwinia amylovora in host plants inoculated by irrigation

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Erwinia amylovora, causal agent of fire blight, is a quarantine bacterium in Europe affecting several economically important rosaceous plants worldwide. It was present in Northern and Central Europe, and in the last 20 years it has spread to Mediterranean countries. Fire blight is one of the most difficult-to-control diseases of pome trees due to the ability of this pathogen to persist in host and non-host reservoirs and spread by different means. Nevertheless, the information about the inoculum sources and dissemination ways of E. amylovora outside host plants such as water is still very scarce.

Recent studies have shown that the fire blight bacterium survives and maintains its pathogenic potential in different types of environmental waters at low and warm temperatures. Then, water could act as a dissemination way of this pathogen. It has been reported that E. amylovora may be disseminated by rain, and irrigation water has been related with fire blight spread in a nursery. However, the transmission of this pathogen by irrigation water has not been determined yet, neither its possible migration from roots to stem and leaves in plants irrigated at soil level. Then, we have studied the ability of E. amylovora to colonize and migrate in plant hosts inoculated by soil irrigation with a green fluorescent protein (GFP)-marked strain, using its wild type strain as control. Bacterial inocula at 107 cfu/ml were used to water one-month pear plants (Pyrus communis cv. Passe Crassane) without wounds or after wounding either the crown or the roots with a needle, using sterile water as negative control. Plants were inoculated either once or every two days up to one week, and incubated at 20°C under quarantine conditions. Pear plants showing fire blight symptoms were sampled at root, stem and leaf level and periodically analyzed for the presence of inoculated strain on nonselective King’s B (plus tetracycline for the GFP-labeled strain) and semi-selective CCT media. E. amylovora-like colonies recovered were identified by a chromosomal PCR. Tissue sections from roots, stems and leaves were further examined by fluorescence microscopy (FM).

Colonization of roots and migration of E. amylovora within the plant did occur after soil irrigation in both wounded and not wounded pear plants, regardless the assayed strain. Further, the pathogen was able to colonize pear roots and to cause blight symptoms in the inoculated plants, even after only one watering. Once inside the host, the pathogen was able to migrate from the roots to the leaves within 2-4 days post-inoculation, with symptom development being initiated mainly in leaves. Most disease symptoms consisted on necrosis in the leaf margins progressing to the whole leaf surface, with exudates in some cases, and lastly necrosis in the stem, but no blight was observed in the roots. Nevertheless, E. amylovora was recovered from roots, stems and leaves of challenged plants. FM examination of different tissues sections revealed interesting data about the influence of inoculum concentration and environmental conditions on GFP-marked strain. Besides colonization and migration abilities of E. amylovora, water dissemination of the fire blight pathogen by soil irrigation is demonstrated for the first time.

In summary, this work raises new concerns on the potential dissemination of E. amylovora by water that should be necessarily taken into consideration to improve preventive and control measures against fire blight.

Keywords: plant pathogenic bacterium; fire blight; water dissemination; pathogenicity; CCT; PCR; fluorescence microscopy; green fluorescent protein.

Colonization pattern of Methylobacterium suomiense CBMB1210 isolated from rhizosphere of and its effect on red pepper growth under greenhouse condition to optimize efficiency of organic manure and lime management

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Pink pigmented facultative methylotrophic bacteria of the genus Methylobacterium possess one or more characteristics of plant-growth promoting traits. On the other the Methylobacterium has been identified as ubiquitous for nitrogen fixation and production of plant growth hormone, stress was given on the colonization pattern of the strain plant root and leaf surfaces visualized by use of green fluorescent marker. Strain M. suomiense CBMB1210, a rhizosphere isolate from rice (Oryza sativa cv. Dong-jin) were obtained by plating the aliquots onto ammonium mineral salts (AMS) medium, with 0.5% methanol as the sole carbon source. The strain was further checked for other growth promoting characteristics like nitrogen fixation, production of indole-3-acetic acid (IAA) and cytoxins.

To facilitate easy monitoring under plant inoculated conditions. M. suomiense CBMB1210 was tagged with green fluorescent protein (gfp) by introducing the plasmid pFAJ1820 by triparental mating. The donor strain E. coli S17-1 (pFAJ1820) and helper strain E. coli HB101 (pRK2013) were grown on LB broth with kanamycin (50 μg ml-1). M. suomiense CBMB1210 the transconjugants were selected on AMS containing 0.5% succinate supplemented with kanamycin 20 μg ml-1 and nalidixic acid 10 μg ml -1. Colonies that showed fluorescence under UV were selected and the presence of gfp gene was confirmed by PCR using YL065 (F) 5’-GCGATGTTAATGGGCAAAAA-3’ and YL066 (R) 5’-TCCATGCCATGTGTAATCCT-3’ primers. Effect of M. suomiense CBMB1210 gfp-29 root elongation was performed in rice and canola plants. Surface-sterilized seeds were kept immersed in the bacterial suspension for 4 h under shaking and then transferred to sterile growth pouches (CYG™ seed germination pouch, Mega International Manufacturer, USA) containing 20 ml sterile distilled water. Seeds treated with sterile water alone was consider as control. The root length of the seedlings from control and M. suomiense CBMB1210 gfp-29 treatments was measured on 7 and 15 days for rice and tomato, respectively. M. suomiense CBMB1210 was strictly aerobic, Gram-negative rods of α-Proteobacteria. The nitrogenase activities of M. suomiense CBMB1210 was 33.2 μmol N2 mg protein -1 h. The strain accumulated the IAA amount 7.04 μg ml-1 in the presence of L-tryptophan while it was 54.78 ng ml-1. Inoculation of M. suomiense CBMB1210 strain increased the root length and dry weight of the seedlings compared to uninoculated control in rice and tomato. M. suomiense CBMB1210 gfp-29, effectively colonized the roots and leaves of rice and tomato when inoculated in the rhizosphere as observed through confocal laser scanning microscope (CLSM) and scanning electron microscopy (SEM). The strain initially colonizing the rhizoplane was able to enter the roots of tomato inter-cellularly. Although, such inter-cellular colonization was not detected in rice, the bacterial cells were present in the leaves and stomata packed with bacteria can be visualized both in rice and tomato. This study put forth conclusively that the rhizosphere soil isolate M. suomiense CBMB1210 has plant growth promotion ability and colonizes the roots and leaf surfaces of potted plants without host speciation and is transmitted to the aerial plant parts from the seed source. Additional green house and field experiments along with optimization of fertilizer management, is in progress to exploit M. suomiense CBMB1210 as biofertilizer. We are looking ahead with M. suomiense CBMB1210 in organic fertilizer and lime use efficiency in red pepper cultivation.

Keywords: Methylobacterium, plant growth, colonization, gfp, rhizosphere, rhizoplane, phylloplane
Comparison of conventional and molecular methods for analyzing soil fungal diversity to determine the impact of soil use purpose

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Soil is very heterogeneous environment and different components of the solid fractions in soil (sand, silt, clay, and organic matter) provide variable microhabitats. Three major factors affecting microbial diversity in the soil are plant type, soil type, and soil management. In some situations the soil and in others plant type is the determining factor affecting the soil microbial community.

Nowadays two approaches are used to analyze soil microbial communities – conventional plating of cultivable microorganisms and molecular methods that are independent of cultivation. Both groups of methods have their own advantages and disadvantages.

The study was undertaken to characterize fungal communities at 12 soil profiles with objective to determine the impact of soil use purpose (forests, abandoned agricultural lands, meadows and arable lands) using conventional plating methods and molecular biology methods. Such conventional methods were used as estimation of the number of cultivable microorganisms and filamentous fungi CFU and the total amount of cultivable microorganisms has the tendency that with increasing depth the amount of microorganisms decreases. The results of the diversity calculations of cultivable microorganisms (CFU) using plate count method and determination of predominant fungal genera and their relative abundance. A robust culture-independent ARDRA and calculations of Shannon-Weaver diversity index, and quantitative PCR with universal fungal primers and Trichoderma spp. specific primers represented molecular methods.

The number of filamentous fungi CFU and the total amount of cultivable microorganisms has the tendency that with increasing depth the amount of microorganisms decreases. The results of the diversity calculations of cultivable filamentous fungi and fungal diversity from ARDRA results have weak correlation (0.41) due to different biases of both methods. The highest Shannon-Weaver diversity index values mostly were in the upper and medium soil layers and the fingerprints of fungal communities changed in different depths of the soil. Forest soils and former agricultural soils generally showed slightly higher diversity of fungi, and the proportion of fungal DNA within total soil DNA was significantly higher than in agricultural soil layers. There is no clear difference between fungal communities in meadows and arable lands. However a tendency can be observed that in arable lands the fungal diversity and proportion of Trichoderma spp. DNA is higher than in meadows.

Keywords: Soil, fungal diversity, Shannon-Weaver diversity index, ARDRA, quantitative PCR, Trichoderma.

Composted olive mill pomace as organic fertiliser in organic olive oil groves

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The amount of olive mill pomace produce annually, during the two-phase extraction procedure, in the Andalusian olive oil industry, in just three-four months, is around 1.0 to 1.5 millions tons. Most (~ 80 %) of the nitrogen (N), phosphorus (P) and potassium (K) taken up with the olives appear in the olive mill pomace during its transformation to olive oil. Till recently olive mill pomace was considered as a residue environmentally harmful. The valorisation of olive mill pomace fulfils some of the objectives of sustainable agricultural systems. Olive mill pomace can be valorised as a potential source of N (P and K) alternative or in combination to, chemical N fertiliser. However, because of the high polyphenol and biological oxygen demand levels and its very high water content, its composting is highly recommended before any further use in agriculture. However, before recommendation on doses, frequency and moment of application of the composted olive mill pomace (COMP), the potential for N, P and K supply must be evaluated.

The objectives of this investigation were evaluated in the short-to-medium term at laboratory and field scales the magnitude and temporal pattern of available N, P and K supplied from COMP during its decomposition and the potential for reduce N lixiviation, by increasing the soil retention capacity. The potential for carbon (C) sequestration was also evaluated.

Decomposition of composted olive mill pomace biologically immobilised N at the short-term (~ 3 mo). N lixiviation in the field was significantly reduced after the application of COMP during one year period independently of the season of its application (autumn or spring). Combination of COMP with a N-enriched (15 % N) accredited organic fertiliser or Urea or nitrate, resulted in a short-term decrease of available N but a slow supply afterward. CO2-COMP-derived during COMP decomposition was low (4 - 7 %) which, together with analysis of the soil C in plots which received COMP in the long-term (17 years), suggest that soil C sequestration after the application of COMP was increased.

The utilisation of COMP as organic fertiliser, not only recycle huge amount of N, P and K but also is an adequate strategy to reduce nitrate lixiviation in many of the olive oil crops area which are included in the nitrate vulnerable zones, and should be taking into account as an strategy to improve C sequestration in olive oil orchards.

Keywords: Composted olive mill pomace, N cycle, C sequestration.
Using agricultural machinery in the field, especially at a high soil moisture, results in soil compaction and hence alteration of soil properties. Evaluating appropriate methods of alleviating the stress of soil compaction on plant growth is of great economical and environmental significance. Data regarding the effects of biological methods such as using arbuscular mycorrhiza (AM) on corn (Zea mays L.) growth under compaction is rare; hence this research work was performed under greenhouse conditions. The objectives were to evaluate: 1) the effects of soil compaction on corn growth, 2) if using different species of AM under non-sterilized and sterilized conditions can alleviate the stress of soil compaction on corn growth. The collected field top soil was sieved and half of it was sterilized using autoclave. The soil was then compacted in 20x20 cm pots using 2-kg weights, planted with corn seeds and inoculated with different species of AM species including Glomus etunicatum, G. mossea, and G. intraradices. Soil and plant parameters including soil resistance to penetrometer and soil bulk density as well as corn root and leaf growth were determined. While high levels of soil compaction decreased corn growth, AM inoculation significantly enhanced root growth and hence plant growth under compaction. These results are very important complementary to the previously rare documented results regarding the effects of AM on corn growth under compaction stress and are of great agricultural and ecological significance.

Keywords: corn (Zea mays L.) root and leaf growth; soil compaction; arbuscular mycorrhizal fungi

Database construction of Basidiomycetes Genetic Resource using ITS region

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Introduction. Wood is attractive renewable material not only as alternative to fossil resource but also as a CO2 absorber. CO2 is absorbed in the cell wall of the wood in the forest and is packed in an urban area for a long term as a wooden material. The expansion of wood demand is effective for the CO2 reduction but is obstructed by biodegradability of wood. Therefore, the pest control for wood conservation is important in wood industry. The research about wood-rot fungi will start from identifying the species of causing fungi. However, the identification of wood-rot fungi is not easy because they lives in the hidden place such as in wood, their mycelia lack recognizable distinction, and morphologic character of their fruit body has a wide variation. The present study therefore aims to construct a database of wood-rot fungi including information about fruit body, colony morphology, collection place, host plant, and short DNA sequence for identification of species. The nuclear internal transcribed spacer (ITS) region was employed as the DNA sequence to identify species. Confirmation of the strain species is essential for reliable database. In the present study, the ITS sequences of the contents strains in this database were determined and then were compared with information in Genbank/EMBL/DDBJ database and the sequences of the strains stocked in other institution.

Materials and methods. The strains of wood-rot fungi were selected from the fungal collection of Deterioration Organisms Laboratory (DOL), Kyoto University. Genomic DNA extracted from fungal colony on potato dextrose agar (PDA) plate. The full-length ITS region was amplified by PCR using primer ITS-1 and ITS-4 and subcloned into the plasmid vector for sequencing. The sequence of each strain was compared with information in GenBank/EMBL/DDBJ database by BLAST search and was checked strain species. More information about species was obtained from cluster analysis.

Results and discussion. The ITS region fragments were amplified from the template genomic DNA extracted from all 114 strains. Biological information, such as nucleotide sequence, is being accumulated rapidly but that about wood-rot fungi is not so sufficient. The enough information to identify species was often not found even if ITS sequence determined newly was searched in the database. In this case, the cluster analysis using the information of related species provided prognostic information. Additional strains about appropriate species obtained from other institute presented contributed to confirm the species. However, the information on the general database involves the risk that submitted information is incorrect and the strain name maintained in institute could have been replaced involuntarily. Both of living strains and sequence information are essential to confirm species.

Keywords: wood-rot fungi, identification, ITS region
Design of xanthomonads-specific molecular markers using CUPID and Insignia

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The genus Xanthomonas comprises many phytopathogenic species of economic relevance. Xanthomonas compestris pv. vesicatoria (Xcv), the causal agent of bacterial spot disease in tomato and pepper, is already disseminated throughout the EPPO (European and Mediterranean Plant Protection Organization) region and strict phytosanitary regulations must be enforced to prevent pathogen spreading and manage infected areas. Molecular-based methods, which are being progressively incorporated into bacteria detection workflows, offer the potential of highly accurate detection and are considered less time-consuming and costly than the established culture-based methods. The efficiency of DNA-based detection methods relies mainly on two factors: the selection of suitable DNA signatures, i.e., stretches of DNA unique to a taxonomic group and absent from all the other, and the selection of an appropriate molecular detection technique [1].

Currently, with more than five-hundred completely sequenced bacterial genomes and access to bioinformatics tools for comparative genomics, discovering suitable DNA signatures is becoming easily accessible for diagnostic microbiologists. In this work, two recent bioinformatics applications, CUPID [2] and Insignia [3], were used for DNA-signature prediction with the aim of designing molecular markers for Xcv. These web-based applications allow retrieving either taxa-specific ORF’s (CUPID) or DNA signatures (Insignia) in a simple and rapid manner. However, in silico specificity tests, using the BLAST tool, showed that some of the calculated regions were not strain-specific. Furthermore, the number of outputted regions in very high, ranging from about 200 ORF’s in CUPID, to more than 200 000 signatures in Insignia. This exceedingly high number of outputted signatures required a thorough data filtering, particularly considering that molecular markers were designed for low throughput detection assays, namely PCR and dot blot hybridization. To achieve a more reliable and convenient signature prediction method, an algorithm was developed to combine the outputs of these two bioinformatics tools. Therefore, the results from CUPID and Insignia were overlapped and the number of DNA loci obtained varied according to the defined minimum sequence length, ranging from 34 loci, for design of small markers (150bp), to 16 loci for design of larger markers (700bp). Primer-pairs were designed for the selected in silico specific regions and their effectiveness was confirmed by PCR using seven different Xcv strains. Uniform PCR conditions were used in all assays and obtained amplicons were cloned and sequenced to confirm their identity. Results showed that six primer-pairs provided consistent amplification with all the target strains. Additionally, the sequence variation of the selected regions in different strains suggested that the six loci are located within conserved and stable genomic regions. Dot blot hybridization assays were used to extensively confirm the specificity of the markers against 16 other Xanthomonas and 24 non- Xanthomonas strains. Positive hybridization signals were observed only for target strains. The most promising markers were applied in PCR or hybridization-based detection trials using infected plant material. A simple and inexpensive sample preparation method, based on crude extract filtration, centrifugation and boiling, was optimized with the intent of developing cost-effective procedures for Xcv detection.

In conclusion, the proposed marker design pipeline allows the output of several DNA-signatures in a simple and rapid manner and can easily be extended to other target species. This work shows the potential of the combined use of CUPID and Insignia to reliably calculate DNA-signatures for any bacterial target of interest.

Keywords: Bioinformatics; CUPID, Detection, Identification, Insignia, Molecular markers, Xanthomonas.

Detection of group I and group II introns in a Mexican Bacillus thuringiensis collection

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Group I and group II introns are self-splicing RNA sequences which are found in bacteria, archaea, mitochondrial and chloroplast genomes. Group I introns work as a ribozyme over another RNA’s while group II introns are ribozymes that are able to excise themselves from precursor mRNA transcripts. They also have ORF for reverse transcriptase enzyme. In this study there analyze a Mexican Bacillus thuringiensis collection obtained by the Instituto de Biotecnología of Universidad Autónoma de Nuevo León. In order to amplify both intron groups, specific primers were designed and we expected PCR products of 600 bp 1965 bp for group I and group II, respectively. All strains, twenty six, were positive for the intron group I. However, four of them gave additional band of 200 bp. Nucleotide sequence of main DNA band revealed higher nucleotide homology with reported sequences. Concern with the group II, thirteen strains amplified a single DNA band of 700 pb, instead 1965 bp. Among these ones, we can find to GM-18 and GM-33 recognized as holotype for the serovar. neoleonensis and monterrey, respectively, who were positive for both kind of introns. Nucleotide sequence of PCR products from group II and the additional 200 bp, will be discussed.

Key words: intron, bacteria, ribozymes.

Determination of soil microbial community fluctuations by different techniques in a maize field

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The soil microbial community in a maize field under conservation agriculture management was analyzed by culture-dependent and culture-independent methods. Of these methods, morphological-type differentiation of cultured microorganisms gave the most detailed and reliable results. Soil PCR-agarose electrophoresis also gave reliable results, but the fluctuations of the different species populations were not detected. Soil PCR-DGGE gave unconvincing results, probably because it has a bias that depends on each particular soil sample and experiment.

Keywords: Soil microorganisms, DGGE, direct count.
Differences Between Microbial Communities in Worm Guts and the Soils They Inhabit

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The soil environment harbours an overwhelming diversity of micro-organisms. Soil microbial communities vary widely in space and, despite considerable interest, there is relatively little understanding of the factors which drive this observed spatial diversity or the variation in soil processes as a result of the differing communities. In addition to these complexities, studies have been hampered by the inability to culture the majority of soil microbial species. However, genome-based molecular techniques, such as pyrosequencing and quantitative PCR, are providing tools to begin to further explore soil biodiversity. Spatial diversity not only varies at a macro level, between differing locations, but also at a micro level, with differences in adjacent location in the same soil: at the surface, in water saturated pockets, at the surface or centre of soil aggregates. Studies of such microvariation will give greater understanding of the correlation between soil taxa and processes.

The gut of the earthworm is a distinct micro-environment, where conditions are markedly different from the soil the earthworm inhabits. Described as a ‘mobile anoxic microzone’ (Drake and Horne, 2007, doi: 10.1146/annurev.micro.61.080706.093139), the midgut is constantly moist and oxygen-free. It is also of stable and relatively neutral pH and rich in water soluble organic material as compared to the soil which the worm inhabits. These anaerobic and high nutrient conditions suggest that microbes active in fermentation and denitrification will be stimulated within the worm midgut.

In this study we investigated differences in microbial fauna in soil from apple orchards under two different production regimes, integrated fruit production (IFP) and organic, in both the bulk soil and within worm midguts. Soil from adjacent orchards in the Hawkes Bay region of New Zealand was placed into twenty litre containers, with a two centimetre thick layer of leaf litter added; twelve earthworms (Lumbricus terrestris) were added to each container. The containers were covered with damp cloth, and kept at a constant 20°C in the dark for three weeks. Soil samples were taken at the beginning and end of the experimental period. Midgut soil and soil from identifiable worm casts were taken at the end of the period. Nucleic acids were extracted from all the soil samples and used to quantify levels of various 16S RNA groups and functional genes. Functional genes selected concentrated on genes within nitrogen metabolism and some facets of carbon metabolism.

Variations between the organic and IFP soils were few and subtle, but between bulk soil and midgut samples had greater differences. Further the variation between the organic soil midgut and bulk soil was greater than that in the IFP samples. The most significant of these were a large decrease in nosZ and smaller increase in nifA in the gut of worms in organic soils as compared to IFP soils. nosZ is part of the enzyme which catalyses the conversion of the potent greenhouse gas N2O to gaseous nitrogen in the final step of denitrification, and the decrease of this enzyme means that earthworms may be causing N2O emission in organic soil. nifA forms part of the enzyme converting nitrite to ammonia, thus removing the first chemical involved in denitrification, converting it into a nitrogen form which is available for plant nutrition. The result of the interaction between these competing processes is unknown.

Keywords: soil microbial biodiversity; earthworm, qPCR, nitrogen metabolism, organic.

Diversity of endophytic fungal community of Vitis labrusca L. (var. Niagara Rosada) and biological control of Fusarium sp. and Botrytis sp.

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Endophytic fungi are those, cultivable or not, that live in the inner plant parts causing no harm to their hosts. These fungi may protect the host plant against diseases and pests and have been considered an important source for bioactive compounds. Previous studies have shown that plants from tropical areas present a great microbial diversity, which could be exploited for biotechnological purposes. The State of São Paulo is the major Brazilian producer of table grape, mainly the Niagara Rosada (Vitis labrusca L.) variety. Therefore, the objectives of the present work were: i) evaluate the fungal endophytic community associated to stems and leaves of V. labrusca cultivated in agro ecological and conventional orchards and ii) evaluate the ability of these endophytic fungi to inhibit Fusarium sp. and Botrytis sp. The endophytic fungal diversity was evaluated by morphological and molecular analysis. The correlation of this genetic and physiological diversity with environmental data was evaluated by Redundancy Analysis (RDA). Endophytic fungi (275 isolates) were consistently isolated from stems and leaves and showed that this community is composed by the Ascomycota and Basidiomycota phylum, being Alternaria, Botryosphaeria, Colletotrichum, Daldinia, Diaporthe, Fusarium, Guignardia, Nodulisporium, Pestalotiopsis, Phoma and Schizophyllum the most frequent genera. The isolation frequency (IF) in leaves was higher than in stems. The IF from leaves of plants cultivated in agro ecological systems was higher than that cultivated in conventional system. Also, the results showed that the temperature was the most important factor on diversity and density of endophytic community of V. labrusca var. Niagara Rosada. Some endophytic fungi were able to inhibit plant pathogenic Fusarium and Botrytis. The present study is the first attempt to reveal the fungi endophytic diversity inside V. labrusca cultivated in Brazil.

Keywords: endophytic fungi; Vitis labrusca; biocontrol, bioactive compounds.
Ecology of coarse wood decomposition by the saprotrophic fungus *Fomes fomentarius*

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Course wood colonised by saprotrophic basidiomycetes represents a unique ecosystem strongly affected by fungal metabolism - low pH, presence of extracellular enzymes of lignocellulose decomposition and large abundance of wood-associated microorganisms [1-2]. The aim of this work was to (1) quantify the activity and spatial distribution of extracellular enzymes in wood and fruit bodies of course wood colonised by the white-rot basidiomycete *Fomes fomentarius* and (2) to analyse the diversity of the fungal and bacterial community in a fungus-colonised wood.

Fruit bodies of *Fomes fomentarius* and adjacent wood of beech (*Fagus sylvatica*) - 15 samples, and birch (*Betula pendula*) - 6 samples, were collected in hardwood forests in the Czech Republic. In addition, the spatial distribution of enzyme activities, fungal biomass and the diversity of fungal and bacterial communities were tested in a log colonised by the fungus. Activities of endo-1,4-

β-glucanase and endo-1,4-β-xylanase were measured with azo-dyed carbohydrate substrates. Fungal biomass was quantified based on ergosterol content. Composition of fungal and bacterial communities was analysed by Denaturing Gradient Gel Electrophoresis.

The comparison of extracellular enzymes distribution in wood and fruiting bodies showed that ligninolytic enzymes were found mainly in colonized wood while the activities in fruit bodies were low. There were also some differences in enzyme production between the two host tree species, the activity of cellulose and xylan-degrading enzymes was significantly higher in beech wood than in birch wood. Spatial analysis of a birch log colonised by the *F. fomentarius* proved that *F. fomentarius* was the only fungal representative found in most samples, except a few where a small amount of other fungi was observed on DGGE gels besides the dominating *F. fomentarius*. In contrary, there was a rich bacterial community that varied with location of the sample. The spatial differences in the composition of bacterial communities in the sample are probably due to the spatial differences in the decay progress. There was a high level of spatial variability in the amount of fungal biomass detected, but no effects on enzyme activities were observed. Samples from the fruiting body showed a high β-glucosidase and chitinase activities compared to wood samples. Samples can be divided into “proximal” and “distal” depending on their distance from the fruiting body as there are significant differences in the amount of measured enzyme activity. Significantly higher relative levels of xylanase, β-glucosidase and cellulohydrolase were found in proximal samples, and higher laccase activity was found in the distal ones. The presence of genes for cellobiohydrolase and laccase were detected in both the fruiting body and wood.

The activity of cellulase and xylan-degrading enzymes was significantly higher in beech wood than in birch wood. Ligninolytic enzymes were found mainly in colonised wood while the activities in fruit bodies were low. Spatial distribution analysis of a birch log revealed differences in enzyme activity distribution between wood adjacent to fungal fruit bodies (higher activity of xylanase, β-glucosidase and cellulohydrolase) and more distant wood parts (higher activity of laccase). Samples from fruiting body show high activity of β-glucosidase and chitinase. The composition of microbial communities (although dominated by *F. fomentarius*) differed among these parts and may depend on enzyme activities. These results show that a significant level of spatial heterogeneity shaping the rate of substrate decomposition as well as the composition of microbial community is present even within a single piece of decaying wood.


**Keywords**: bacteria; basidiomycetes; environmental microbiology; enzyme; fungi; lignocellulose; microbial ecology; wood microbiology

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Effect of PGPR with AMF on tomato lycopene and antioxidant content

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Tomato, is today the most popular garden vegetable in the world and excellent source of lycopene, which is the pigment that makes tomatoes red and has been linked to the prevention of many types of cancer. Lycopene is an antioxidant which fights free radicals that can interfere with normal cell growth and activity.

Greenhouse experiment was set as follows: tomato (*Lycopersicon esculentum* F1 Hybrid, GS-15) from seeds after inoculated with bacteria (pgpr) and mycorrhiza (AMF) were grown in mixed of soil field, waterworn sand and peat (1/3, v/v each of them), after three weeks seedlings transfer to pots with 7 kg soil (mixed). there were 7 treatments for bacteria (*pseudoomonas, azotobacter, azospirillum, pseudomonas + azotobacter, pseudomonas + azospirillum + azotobacter + azospirillum*) and two treatment for AMF (*with AMF and without AMF*). AMF was mixed of (*Glomus intraradics + Glomus mossea + Glomus etanicatum*). For comparison set control 4 pots. Lycopene and antioxidant in fruit were determined.as a result:

In all treatments lycopene and antioxidant were higher than the control treatment.

Mamimum lycopene content in different bacteria levels related to mixed of tree bacteria (90.42 mg/kg fresh weight) and in different bacteria × AMF levels related to mixed of tree bacteria with AMF treatment (98.30 mg/kg fresh weight), it showed a positive interaction of bacteria and AMF on tomato lycopene content. Also maximum antioxidant content in different bacteria levels related to mixed of tree bacteria and in different bacteria × AMF levels related to mixed of tree bacteria with AMF treatment (54.65%). But minimum content of antioxidant was in pseudomonas + azotobacter treatment (40.03%) whereas when AMF added to this treatment the antioxidant activity increased (47.93%). that show the AMF can decrease the negative interaction effect of pseudomonas + azotobacter activity on tomato antioxidant content.

A positive relation was seen between lycopene content antioxidant in all treatment (r=89%).

**Keyword**: lycopene, antioxidant, pseudomonas, azotobacter, azospirillum, AMF

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Effect of *Pseudomonas* and *Azotobacter* with mycorrhiza on Two varieties of tomato plant growth

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Many marketable biofertilizers are mainly based on plant growth-promoting rhizobacteria (PGPR) that exert beneficial effects on plant development often related to the increment of nutrient availability to host plant (Verschelde, 2003). Among the symbiotic microorganisms, arbuscular mycorrhizal fungi (AMF) form mutual associations with more than 80% of plant species. Benefits to plants include improved mineral nutrition (Smith and Read 1997), protection against pathogens (Azcon-Aguilar et al. 2002) and enhanced resistance or tolerance to stress (Turnau and Haselwandter 2002).

Glasshouse experiments were conducted to assess the influence of *Pseudomonas putida* strain 41, *Azotobacter chroococcum, Pseudomonas putida* strain 41 + *Azotobacter chroococcum alone and with arbuscular mycorrhizal fungus mixed (Glomus mossea) on growth of tomato varieties (*lycopercicum esculentum* var. Tivi F1 and *lycopercicum esculentum* var. Delba F1) in control treatment without bacteria and AMF was considered. Added N fertilizer to all treatments. Shoot dry weight, root dry weight, total plant dry weight, root dry weight / shoot dry weight, root dry weight / total plant dry weight, concentration of K, P, Mg and Ca in plant shoots were the factors that measured in treatments. As a result, It was significant difference between interaction effects of bacteria and fungi and varieties on nutrient content of two varieties, and in var. Tivi F1, was higher than var Delba F1, *Pseudomonas putida* strain 41 + AMF treatment had maximum of nutrient content in two varieties. *Azotobacter chroococcum alone treatment had minimum of nutrient content in two varieties.

*Pseudomonas putida* strain 41 + *Azotobacter chroococcum* with AMF treatment had maximum of shoot and root dry weight in two varieties, and in var. Tivi F1, was higher than var Delba F1, but was not significant difference between them. All factors in control treatment were lower than the other treatments.

It is peer that use of these biofertilizers can be effective on var. Tivi F1 tomato plant growth.

Keyword: tomato, *Pseudomonas*, *Azotobacter*, varieties, AMF, growth, nutrient

Effect of biofumigation with manure amendments and repeated biosolarization on *Fusarium* densities in pepper crops

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In the region of Murcia (Southeast Spain), sweet pepper has been grown as a monoculture in greenhouses for over twenty years. Soil has usually been disinfected with methyl bromide to control pathogens and to prevent soil fatigue effects until its banning in 2005. Populations of *Fusarium* spp. seem to be related with decline in sweet pepper monoculture performed without soil fumigation, in the absence of soil-borne pathogens. In this study, soils were treated with manure amendments, alone (biofumigation, B) and in combination with solarization (biosolarization, B+S), with or without the addition of plant residues. A gradual decrease in the amount of manure amendment in the case of biosolarization was also evaluated. The treatments were compared with methyl bromide. Experiments were conducted at two different locations, one for three years and another for only one year. Disinfection effects were measured by the density of *Fusarium* spp. isolated from soil before and after fumigation. A soil-dilution plate method with Komada medium was used for isolating *Fusarium*. Three different species were systematically isolated: *Fusarium oxysporum, Fusarium solani* and *Fusarium equiseti*, being *F. solani* the most abundant one before and after the treatments. The repeated use of manure amendments with crop residues, without solarization, was not able to decrease the *Fusarium* spp. density relative to methyl bromide-treated soil. However, the effectiveness of biosolarization (with or without adding plant residues) and its repetition - reducing the rate of the amendment - for control of *Fusarium* spp. populations was the same as or even greater than that of methyl bromide.

Keywords: *Fusarium* spp.; manure amendments; biosolarization; pepper
Effect of DFO-B siderophore on lead sorption by Na-montmorillonite

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Siderophores are important chelators influencing bioavailability and fate of heavy metals in soils. They are low molecular weight organic ligands excreted by aerobic soil microorganisms (fungi and aerobic and facultative anaerobic bacteria and some plant roots) to acquire Fe. Siderophores may also complex other metal cations such as Pb. This ability to form stable heavy metal siderophore complexes suggests that these ligands may affect heavy metal bioavailability and mobility in soils. The main objective of this study was to investigate the effects of desferrioxamine B (DFOB) siderophore on sorption of Pb on sodium saturated montmorillonite. The sorption of Pb on montmorillonite was studied as a function of pH (sorption envelopes) and as a function of Pb concentration (sorption isotherms) in the presence of siderophore using a 24h batch equilibration experiment. The sorption envelopes (Figure 1) showed that the siderophore increased sorption of Pb onto montmorillonite at all solution pH, specially at pH ≥5, by 76-97%. This increasing in the presence of siderophore was likely due to sorption of the positively charged H₂Pb(DFOB) and H₂Pb(DFOB)⁺ complexes to negative charges of the mineral.

![Figure 1: Sorption of Pb as a function of pH in the presence and absence of DFOB siderophore (H₂Pb(DFOB) and H₂Pb(DFOB)⁺)](image)

Adsorption isotherms revealed that removal of Pb from solution was not affected by siderophore at pH = 4.5, while the siderophore strongly influenced the isotherm shape and increased Pb sorption on montmorillonite at pH = 6.5. The equilibrium data were well described by a Freundlich isotherm, in which the values obtained for the Kf constant were 4.8 and 197 Lg⁻¹ and for n constant were 0.8 and 2.1 in the absence and presence of the siderophore, respectively. These values are indication of high sorption affinity of Pb on montmorillonite in the presence of the siderophore. Our results showed that DFOB siderophore can strongly affect Pb sorption on clay minerals. Thus DFOB siderophore is expected to increase Pb sorption to negatively charged soils and may reduce Pb toxicity to plants and leaching to ground waters.

Keywords: Lead, Siderophore, Sorption, Montmorillonite

Effect of different rhizobia on Pfallar (Phaseolus lunatus L.) in the Valley of Nazca in Peru

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Pallar, also known as Lima bean, is the most important grain legume in the Ica region located at the southern coast of Peru where it has got ‘the origin of denomination’ [1]. This legume represents an important cultural legacy, since its prehispanic origins, and a great source of protein for the Peruvian population. It can establish symbiosis with certain soil fixing nitrogen bacteria broadly known as rhizobia. The aim of the present study was evaluate the effect of inoculation of eight strains of Rhizobium sp. and Bradyrhizobium sp. on pallar seeds (Phaseolus lunatus L. var. Generoso de Ica) at field conditions. The experiment was set in the Ingenio Valley in the province of Nazca of the Ica region. Relative humidity was 85% and maximum and minimum temperatures were 30.8 and 7.4 °C, respectively. Soil was sandy loam, slightly alkaline, fairly saline, with low organic matter content and phosphorus and potassium average availability.

The strains were isolated from nodules of pallar fields of Ica Valley, characterized and selected previously at the LEMYB of the UNALM [2, 3]. These were finger-printed with BOX-PCR and sequenced with rD1 and fD1 rDNA 16S gene primers. The field experiment was design with Randomized Complete Block design (RCB), with 10 treatments and 4 repetitions. Nitrogen treatment was fertilized with NPK mixture, while the others were just fertilized with P and K. Two evaluations were done, one during flowering (nodulation, dry weight and N percentage of the aerial part) and the other at harvesting (agronomic characteristics like number of grains/pod, yield by plant and plot, 100 grains weight and seed N percentage).

Different BOX profiles were found in all the strains and the bradyrhizobia were identified as B. yuanmingense. During flowering, all strains promote the growth of the crop against the controls, whereas there were not significant differences. The inoculated plants showed nodules much more red and big than the non-inoculated ones. At harvesting, differences in the agronomic characteristics were significant between inoculated and non-inoculated treatments (Table 1). Strains of B. yuanmingense LMTR 56030, LMTR 28 and LMTR 56010 were the most effective on pallar. These results showed the great potential of these bacteria as biofertilizers and its consequent use in an environmentally friendly agronomic management practices.

Acknowledgments: INCAGRO Sub-proyecto 2007-548; Asociación de Agricultores de Ica and FDA 111-biol/UNALM.

Keywords: Rhizobium sp.; B. yuanmingense; P. lunatus; effectivity; inoculation.

Table 1: Effect of strains of Bradyrhizobium on the yield of pallar

<table>
<thead>
<tr>
<th>Treatments</th>
<th>N° of pots</th>
<th>100 grains wt (g)</th>
<th>Yield/plant (g/plant)</th>
<th>Yield/plot (Kg/plot)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1: LMTR 56004</td>
<td>149.50 bcd</td>
<td>202.84 bcd</td>
<td>420 bc</td>
<td>10.086 bc</td>
</tr>
<tr>
<td>T2: LMTR 56009</td>
<td>147.04 abc</td>
<td>199.17 abcd</td>
<td>434.5 bc</td>
<td>10.433 bc</td>
</tr>
<tr>
<td>T3: LMTR 5610</td>
<td>160.44 abc</td>
<td>207.52 abc</td>
<td>496.75 abc</td>
<td>12.055 ab</td>
</tr>
<tr>
<td>T4: LMTR 5617</td>
<td>154.46 abcd</td>
<td>203.50 abcd</td>
<td>447.5 bc</td>
<td>10.759 bc</td>
</tr>
<tr>
<td>T5: LMTR 56025</td>
<td>142.75 abc</td>
<td>205.67 abcd</td>
<td>429 bc</td>
<td>10.326 bc</td>
</tr>
<tr>
<td>T6: LMTR 56026</td>
<td>157.38 abc</td>
<td>207.55 abc</td>
<td>482 abc</td>
<td>10.816 bc</td>
</tr>
<tr>
<td>T7: LMTR 56030</td>
<td>178.21 a</td>
<td>213.99 a</td>
<td>540 a</td>
<td>13.454 a</td>
</tr>
<tr>
<td>T8: LMTR 28</td>
<td>171.21 ab</td>
<td>204.50 abcd</td>
<td>490 ab</td>
<td>11.766 ab</td>
</tr>
<tr>
<td>T9: N⁺</td>
<td>151.75 b</td>
<td>219.92 ab</td>
<td>456.5b c</td>
<td>10.953 bc</td>
</tr>
<tr>
<td>T10: N⁻</td>
<td>132.56 a</td>
<td>197.13 d</td>
<td>379.73 e</td>
<td>9.18 b</td>
</tr>
</tbody>
</table>

Values followed by the same letter do not differ at p<0.05, according to Duncan test.

References:
Effect of different rhizospheric bacteria in the growth of *Gossypium barbadense* L. in Perú

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The economical development in Peru is hardly anchored to the cotton. Because it is one of the main activities in the region which is used in the oil, textile and cattle industries. As a result, more than 20 thousand families depend on this crop. Therefore, nowadays new tendencies in agriculture like sustainable management and possibilities of some microorganisms like the plant growth promoting rhizobacteria (PGPR) are being opened in order to research about new alternatives in cotton. Working on this topic, the objective of the current study was research the effect of *Bacillus* sp., *B. yuanmingense* and *Azotobacter* sp. strains in cotton (*Gossypium barbadense* L. var. Tangtis) in laboratory and field conditions.

The selected strains from Ica soils were previously isolated in the LEMYB-UNALM [1]. In the laboratory assay, 13 treatments with 6 repetitions were used, 2 cotton seeds were inoculated with 1 ml of 10^7 cell/ml seed. 0.05% KN03 was applied in the N+ treatment. In the field assay, 3 strains selected in the laboratory assay and their combinations were studied in Ica fields. A Randomized Blocks Design (RBD) with 7 treatments and 4 repetitions were used. The N+ treatment were fertilized with NPK mixture while the others were only fertilized with P and K. The seeds were pelleted with a 10^7 cell/ml population. The range of soil temperature was between 21.5 and 27.4 ºC and the minimum and maximum of environment temperature was between 9 and 34ºC, respectively. The plant height was analyzed and the leaf color at the flower buds stage. The microbial activity was determined through microorganisms respiration by quantification of the CO2 production [2].

The interaction *B. yuanmingense* LMTZ 28 - *Azotobacter* sp. LMTZ 56554b was one of the best treatments against the control non-inoculated (N-). This interaction showed the highest values in dry and fresh weight in the aerial part in the laboratory and plant height in field assay. Oddly, in the field, this treatment had an early flowering. Besides, the best microbial activities were obtained with the treatments with *Azotobacter* sp. LMTZ 56554b, *B. yuanmingense* LMTZ 28 and the interaction of both. These results showed that the former interaction has a great potential as biofertilizers and then it could have an important role in a sustainable management program. Acknowledgements: Grant PROTEC 249-2008-CONCYTEC-OAJ, FDA 111-biol-UNALM, Asociación de Agricultores de Ica.

Keywords: *Bacillus* sp., *Azotobacter* sp.; *B. yuanmingense*; *Gossypium barbadense* L.; microbial activity; inoculation.

Table 1. Dry weight and fresh weight in laboratory plants, Height and microbial activity from field essays.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Microbial Activity (mg CO2/g h) (field)</th>
<th>Plant height (cm) (field)</th>
<th>Fresh weight (g) (laboratory)</th>
<th>Dry weight (g) (laboratory)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Azotobacter</em> sp. LMTZ 56554b</td>
<td>0.0900</td>
<td>64 a</td>
<td>0.840 ab</td>
<td>0.1267 ab</td>
</tr>
<tr>
<td><em>Bacillus</em> sp. LMTB 56013</td>
<td>0.0675</td>
<td>49.5 a</td>
<td>0.763 a</td>
<td>0.1067 a</td>
</tr>
<tr>
<td><em>B. yuanmingense</em> LMTZ 28</td>
<td>0.0828</td>
<td>59.5 a</td>
<td>0.858 ab</td>
<td>0.1280 ab</td>
</tr>
<tr>
<td><em>Azotobacter</em> sp. LMTZ 56554b + <em>B. yuanmingense</em> LMTZ 28</td>
<td>0.0799</td>
<td>63.5 ab</td>
<td>0.973 bc</td>
<td>0.1450 ab</td>
</tr>
<tr>
<td><em>Bacillus</em> sp. LMTB 56013 + <em>B. yuanmingense</em> LMTZ 28</td>
<td>0.0617</td>
<td>78 b</td>
<td>0.787 ab</td>
<td>0.1217 ab</td>
</tr>
<tr>
<td>N-</td>
<td>0.0665</td>
<td>63 ab</td>
<td>1.120 c</td>
<td>0.1667 b</td>
</tr>
<tr>
<td>N+</td>
<td>0.0572</td>
<td>54 a</td>
<td>0.748 a</td>
<td>0.1200 a</td>
</tr>
</tbody>
</table>

Values followed by the same letter do not differ at p<0.05, according to Duncan test.

References:
Zúñiga, D. 2009. Uso de Bacterias Promotoras de Crecimiento vegetal para la produccion orgánica de cultivos nativos de algodon y palma en el Valle de Ica. Informe Proyecto PROTEC 249-2008-CONCYTEC-OAJ.

Effect of essential oils on decay resistance of wood

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Despite the wide use of essential oils in pharmaceutical and food industry as antimicrobial agents, their use as wood preservatives has not been fully explored. In this study, essential oils were first screened in nutrient medium for their antifungal activity against common wood inhibiting fungi. Subsequently, one essential oil, eugenol was evaluated for wood durability testing using radiata pine sapwood blocks against 3 common wood decaying fungi.

During the initial in-vitro screening trial, variability in the tolerance of the tested fungi towards the selected essential oil was apparent. Some of the essential oils such as geranium, cinnamon leaf and eugenol completely inhibited the growth of all test fungi at 0.5% w/v on nutrient medium, whereas, eucalyptus oil tested in this study was unable to restrict the growth of any test fungi even at 1% w/v concentration.

Wood durability test results on radiata pine confirmed the antifungal activity of eugenol but highlighted the leachibility (when exposed to wet conditions) of this compound from wood. Blocks treated with 3% w/v eugenol without a leaching cycle had less than 1% weight loss when exposed to all three tested wood decaying fungi, *Coniophora puteana* and *Fomitiporia mediabolistic* while blocks which were leached showed weight losses in the range of 13.40 to 23.12%.

However, blocks which were leached showed weight losses in the range of 13.40 to 23.12%.

This study identified eugenol as a potential benign wood preservative for treatment of timber not exposed to severe leaching, e.g. New Zealand hazard class H1.2. However, to be used for higher decay hazard situations, further work for in-situ polymerization of eugenol to fix active(s) in wood is underway.

Key words: decay resistance test; eugenol; in-situ polymerisation; radiata pine; wood degrading fungi.

Acknowledgements: Grant PROTEC 249-2008-CONCYTEC-OAJ, FDA 111-biol-UNALM, Asociación de Agricultores de Ica.
Effect of in vivo passage on germination and virulence of entomopathogenic fungi, *Verticillium lecanii*

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Information on the effect of repeated sub-culturing as well as passage of the pathogen through its host and laboratory storage on spore viability and virulence of entomopathogenic fungi are essential cardinals for using in biological control programmes. This experiment was conducted to determine the effect of serial passage on virulence of *Verticillium lecanii*. The pathogen was passed through *B. brassicae* for 10 generations and after each passage, virulence of re-isolated fungus was tested on the aphid. Simultaneously, the fungus was re-cultured from the stock culture on PDA medium.

During the first five passages of *V. lecanii* through *B. brassicae* the germination of fungal spores showed no significant difference. At 6th, 7th and 9th passages spore germination slightly increased (93.17-94 percent). Maximum spore germination (94.33 percent) was observed at 9th and 10th passages which was significantly higher than other generations. Germination of spores without in vivo passage showed significant decline at 2nd generation. From 4th generation to 7th generation, spore germinations were on par and significant decrease again was observed from 8th generation. Aphid mortality caused by the fungus was enhanced by serial passing the fungus through the aphid. Aphid mortality after 2nd and 9th passages significantly increased. Mortality caused by the fungus without passing through the aphid significantly decreased in 4th, 5th, 7th, 8th and 9th generations of sub-culturing.

The results indicated a decrease in spore germination and aphid mortality without in vivo passage. On other hand, for mass culturing, the fungus should not be sub-cultured for more than 3 generations without in vivo passage. In vivo passage through the aphid resulted in increased spore germination and aphid mortality after 2nd passage.

**Keywords:** entomopathogenic fungi, *Verticillium lecanii*, in vivo passage, virulence


Effect of preservation methods on *Beauveria bassiana* viability

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*Beauveria bassiana* is an important entomopathogenic fungus (EF) that has been currently used as a biocontrol agent of insect pests. Maintaining and preserving *B. bassiana* cultures is essential for the effective evaluation of its potential as microbial agent against insect pest, for biodiversity studies and also for exchange of fungal material between laboratories. There are many possible methods for the preservation of fungal cultures. However, none of them could be universally applied to all fungi. In the present work we intended to evaluate the suitability of different storage methods, in order to maintain the viability of *B. bassiana* isolates. Fungal isolates were obtained from naturally infected *Prays oleae* pupae, collected in different olive groves in the Trás-os-Montes region (Northeast of Portugal). Three isolates were selected and their conidia were stored, either in 30% (v/v) glycerol at -20ºC or lyophilized. Subculture on PDA medium was used as control. After one year of storage the vegetative growth, sporulation, spore germination and morphological characteristics of each fungal culture were assessed. The results obtained showed that *B. bassiana* viability depended on both storage method and isolate. Cultures of all isolates were growth after one year of storage in 30% (v/v) glycerol at -20ºC, whereas in freeze-drying isolates only two were growth. The number of conidia produced by isolates was significantly lower on cultures preserved by lyophilisation, when compared to cultures preserved in glycerol. However, no significant differences were found on the percentage of spores germinated between the several storage methods tested. Also, there were no macroscopic nor microscopic alterations in mycelial morphological characteristics between isolates preserved in the different storage methods. From these results, freezing at -20ºC seems to be the best storage method for *B. bassiana*. However, the viability of the isolates was probably more strain-specific than dependent on the preservation technique used.

**Keywords** Fungus stock preservation, viability, *Beauveria bassiana*, entomopathogenic fungal.
Effect of the irrigation by worn water on some physiological and biochemical parameters of the bread wheat (Triticum aestivum L.) in the region of Guelma (Algerian East)

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A test on bread wheat was led in order to check the effects of worn water on some physiological and biochemical parameters. Two treatments were chosen, irrigation by worn water, compared to a check. The tests concerned the chlorophyll content, soluble sugar and proline. As far as the obtained results are concerned, the total chlorophyll content has been superior in the treated plants. This shows the ability of plants to react favourably under worn water irrigation. The soluble sugars, were often taken as reference’s tolerance, to abiotic stress, were accumulated more than at leaves and roots level of the treated plants. The content of proline at the leaves and roots of the treated varieties were superior to check, leading to the probable explanation that there is an ability of the cultivars to sustain abiotic conditions. Even though the results that have been obtained are somewhat positive in the expression of the varieties, awareness has to be considered. Numerous studies and experiments have permitted these last decades, to establish standards more and more precise when it comes to deal with worn water in agriculture purpose.

Keywords: Worn water, Chlorophyll, Soluble sugar, Proline, Stress, bread wheat, Tolerance, Semi-arid zone.

Effects of PGPR on tomato plant growth and nutrients uptake

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Tomato, is today the most popular garden vegetable in the world. Tomatoes are high in Vitamin A and C and are naturally low in calories. Inoculated with plant-growth promoting rhizobacteria (PGPR) has been attributed to the production of plant growth regulators at the root interface, which stimulated root development and resulted in better absorption of water and nutrients from the soil (Kloepper et al., 1991; Zimmer et al., 1995; Höfl ich and Ku‘hn, 1996).

A greenhouse experiment was set as follows: tomato (Lycopersicon esculentum Red Cherry) plants were grown from seeds after inoculated with bacteria. There were 7 treatments for bacteria ( pseudomonas, azotobacter, azospirillum , pseudomonas + azotobacter , pseudomonas + azospirillum , azotobacter + azospirillum and pseudomonas + azotobacter + azospirillum ) which compared to control. Plants were cut at prebloom stage.

Maximum level of shoot fresh weight was shown on azotobacter +azospirillum , pseudomonas + azotobacter + azospirillum and azosprillum treatments which significantly differ from other treatments. Maximum level of root fresh weight was achieved in azotobacter +azospirillum , pseudomonas + azotobacter + azospirillum and azotobacter treatments which significantly differ from other treatments. Maximum level of shoot and root dry weights were shown on azotobacter +azospirillum and pseudomonas + azotobacter + azospirillum treatments which significantly differ from other treatments. Maximum root length was shown on azotobacter +azospirillum which significantly differ from other treatments.

The highest amount of N,P and K were shown on pseudomonas + azotobacter + azospirillum treatment which significantly differ from other treatments and the lowest amount was shown on pseudomonas + azotobacter treatment. Maximum level of Ca and Mg were achieved on pseudomonas + azotobacter and pseudomonas + azospirillum treatments which significantly differ from other treatments.

Keyword: tomato , nutrient uptake , pgpr, growth
Effects of *Pseudomonas*, *Azotobacter* and *Azospirillum* on tomato potassium content and fruit quality

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b Soil and Water Institute, Tehran, Iran.  
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The tomato (*Solanum lycopersicum*, syn. *Lycopersicon lycopersicum* & *Lycopersicon esculentum*) is a herbaceous, usually sprawling plant in the Solanaceae or nightshade family that is typically cultivated for the purpose of harvesting its fruit for human consumption. Tomato consumption has recently been demonstrated to be beneficial to human health, because of its content of phytochemicals such as lycopene, β-carotene, flavonoids, vitamin C, total soluble solid (TSS) and many essential nutrients.

A greenhouse experiment was set as follows: tomato (*Lycopersicon esculentum* F1 Hybrid, GS-15) from seeds after inoculated with *pseudomonas*, *azotobacter*, *azospirillum*, *pseudomonas + azotobacter*, *pseudomonas + azospirillum*, *azotobacter + azospirillum* and *pseudomonas + azotobacter + azospirillum*). For comparison set control 4 pots. Lycopene and antioxidant in fruit were determined. As a result:

Minimum shoot potassium and fruit potassium content were seen in *pseudomonas + azotobacter* (respectively, 5.95% and 2.82%) and *pseudomonas + azospirillum* (respectively, 5.67% and 2.72%), treatments, that had significant difference with other treatments. Shoot potassium content of other treatments had no significant difference, however, *azotobacter + azospirillum* treatment had maximum shoot potassium content (7.21%). Minimum fruit antioxidant activity content had related to *pseudomonas + azotobacter* treatment (40.02%). Maximum and minimum fruit lycopene content related to *azotobacter + azospirillum* (67.72 mg/kg fresh fruit) and *pseudomonas + azospirillum* (43.02 mg/kg fresh fruit) respectively. Maximum fruit total soluble solid (TSS) found in *azotobacter + azospirillum* (6.89%) and minimum found in *pseudomonas + azospirillum* (5.93%) treatments. A positive relation was seen between shoot potassium content with antioxidant activity, lycopene and TSS in all treatments.

It showed that there was a negative interaction between effect of *pseudomonas* with *azotobacter or azospirillum* on potassium uptake and fruit quality of tomato.

Keywords: lycopene, antioxidant, *pseudomonas*, *azotobacter*, *azospirillum*, TSS, potassium.

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Effects of drought stress and arbuscular mycorrhiza on maize (*Zea mays L.*) growth characteristics.

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A pot experiment was conducted to investigate the influence of arbuscular mycorrhizal (AM) fungus on drought tolerance in tropical maize. This experiment conducted as factorial based on completely randomized design. The arbuscular mycorrhizal species include: *G.clarideum, G.spinosa, G.intraradices, G.mosseae, G.geosporum, G.hoi*, and control (no inoculated plants). Maize plants were subjected to drought stress for two months. Drought stress levels were: 100% FC, 66% FC and 33% FC as normal irrigation, medium and severe stress, respectively. Drought stress significantly decreased plant height, shoot fresh and dry weight, leaf number and leaf area, and with increase in drought stress, these traits significantly decreased. These traits were also significantly higher in mycorrhizal plants compared control. The ranking of arbuscular-mycorrhizal fungal effects on drought tolerance, based on the relative decreases in shoot dry and wet weight, was as follows: *G.intraradices > G.mosseae > G.clarideum > G.spinosa > G.geosporum > G.hoi > control plants.*

Keywords: drought stress, arbuscular mycorrhiza, maize (*Zea mays L.*)
Endophyte screens from Taiwan native Anoectochilus formosanus Hayata roots.

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The Anoectochilus formosanus Hayata (Figure 1) is a perennial terrestrial orchid that belongs to the family Orchidaceae. It is a well known folk medicine that has been reported for treatments in cancer, hypertension, diabetes and hepatitis in Taiwan and several other nations. In addition to their medicinal properties, A. formosanus Hayata has been manufactured into tablets, capsules, and teas for health food purposes. Because of its high value, these plants have been destroyed from the nature in a tremendous speed for the benefits they provide. Therefore, the production of A. formosanus Hayata at present involves tissue culture and facility cultivation. After tissue culturing, A. formosanus Hayata is planted in pots and acclimated in an open environment facility during which cast great challenge to farmers as it is susceptible to various diseases that decrease plant growth, biomass, and survivability. Most common diseases are Fusarium spp., Phytophthora spp., Pythium spp., Erwinia spp. etc., and they have been reported to cause great losses in A. formosanus Hayata production. In many cases, farmers apply chemical fungicides and bactericides to treat disease infested plants. This kind of management is not effective and will definitely increase consumers doubt on the safety of the product. In order to develop a natural biological control method to manage A. formosanus Hayata diseases, we have collected native A. formosanus Hayata from Ta Hsueh Shan Mountain in attempt to screen for rhizosphere and endophytic microorganisms that can antagonize common diseases of A. formosanus Hayata to improve disease resistance and yield.

Roots of native A. formosanus Hayata were washed with sterile water, surface sterilized with 1% NaOCl for 5 min, and rinsed with sterilized water for several times. Then, roots were ground by sterile mortar and spread on nutrient agar by serial dilutions method. Growth of microbes was observed after 3-5 days in a 25°C incubator. Each microbe was purified, cultured and their DNA purified for 16S rRNA gene sequencing reactions for positive identification. The microbes identified thus far are summarized in Table 1. It is interesting to note that several of these microbes have been reported to be psychrotolerant, which may reflect the natural habitat of A. formosanus Hayata at high altitude. In addition, Bacillus megaterium has also been reported to inhibit the mycelial growth of Rhizoctonia solani that causes lily root rot. Further studies on these microbes will include confirmation of endophytism, pathogenicity tests, antagonistic interaction towards common diseases of A. formosanus Hayata, and their growth effects on A. formosanus Hayata in the presence of pathogenic strains. These experiments will enable us to develop an effective biological control reagent to improve disease resistance and increase A. formosanus Hayata yield in the field.

Keywords: Anoectochilus formosanus Hayata; endophytic microorganism; biological control; disease resistance.

Table 1. Microbes isolated from roots of Anoectochilus formosanus Hayata.

<table>
<thead>
<tr>
<th>Bacterial isolates</th>
<th>Closest match according to the 16S rRNA gene sequence</th>
<th>Similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>THS-B14</td>
<td>Bacillus megaterium</td>
<td>99.5%</td>
</tr>
<tr>
<td>THS-B9</td>
<td>Bacillus odyseyi</td>
<td>99.4%</td>
</tr>
<tr>
<td>THS-B6</td>
<td>Bacillus subi</td>
<td>100%</td>
</tr>
<tr>
<td>THS-B11</td>
<td>Bacillus weihenstephanensis</td>
<td>100%</td>
</tr>
<tr>
<td>THS-B23</td>
<td>Bacillus psychrodurans</td>
<td>99.0%</td>
</tr>
<tr>
<td>THS-B15</td>
<td>Bacillus mycoides</td>
<td>99.8%</td>
</tr>
<tr>
<td>THS-B2</td>
<td>Bacillus cerebensis</td>
<td>100%</td>
</tr>
<tr>
<td>THS-B17</td>
<td>Vibribacillus arvi</td>
<td>100%</td>
</tr>
<tr>
<td>THS-B9</td>
<td>Lysinibacillus boronotoleran</td>
<td>99.0%</td>
</tr>
<tr>
<td>THS-B13</td>
<td>Lysinibacillus sphaericus</td>
<td>100%</td>
</tr>
<tr>
<td>THS-B13'</td>
<td>Lysinibacillus fusiformia</td>
<td>100%</td>
</tr>
</tbody>
</table>

Endophytic bacteria associated with tropical mangrove forests: characterization and biotechnological applications

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Mangrove ecosystems are rich in organic matter, where microbial activity is responsible for major nutrient transformations and the microorganisms play important role in the nutrient recycling of the mangrove forest. Endophytic microorganisms are those that live inside of a plant at least in a period of its vital cycle, and are found in tissues such as leaves, branches and roots. Apparently, they do not cause any damage to the host, which distinguishes them from the phytopathogenic microorganisms. The aim of this work was to study the diversity of endophytic bacteria from typically plant species from mangrove, such as Rhizophora mangle, Avicennia nitida and Laguncularia racemosa, by culture-dependent and denaturing gradient-gel electrophoresis (DGGE). Also, the evaluation of enzyme production such as amylase, esterase, lipase, protease, pectinase and cellulase, by the bacteria isolated from mangrove was tested, aiming a biotechnological potential aspect. The predominant main genera of endophytic bacteria found were Methylobacterium, Bradyrhizobium, Novosphingobium, Sphingomonas, Flavimonas, Microbacterium, Xanthomonas, Stenotrophomonas, Pantoea, Klebsiella, Salmonella, Escherichia and Enterobacter. The analyses by DGGE (Figure 1) showed similarity of bacteria communities from endophytic groups. The results suggest that DGGE is a practicable protocol to assess the complex endophytic bacteria community of mangroves. As for the biotechnological potential of this endophytic community, the isolates were able to fix nitrogen (BNF), to synthesize IAA, and to solubilize phosphate. Also, the isolates presented enzymatic activity, which were ranked as follows: proteolytic (69%), amilolytic (56%), lipolytic (9%), esterasic (47%), pectinolytic (75%) (Figure 2). Cellulolytic activity was not detected.

Keywords: biodiversity, DGGE, BFN, IAA, phosphate, enzymatic analysis.

![Figure 1. Native Anoectochilus formosanus Hayata collected from Ta Hsueh Shan Mountain.](image1)

![Figure 2. Enzymatic production (lipase and esterase) by endophytic bacteria isolated from mangrove species.](image2)
**Entomopathogenic fungi to control the cherry fruit fly *Rhagoletis cerasi* Loew (Diptera: Tephritidae) in Shahrood region, northeast of Iran**

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Cherry fruit fly is the most important pest of cherries in Shahrood region. Without treatment up to 100% of the fruits can be infested. Pest control against the cherry maggot is difficult because of cherry fruits ripening time and long life of insecticides. For long years broad spectrum and acute toxic insecticides, mainly organophosphates, have been used for its control that resulted in contamination of the fruit and the environment. The use of micro-organisms as biological control agents against *R. cerasi* might be an alternative. The aim of this study was to investigate the occurrence of entomopathogenic fungi (EPF) in cherry orchards for selecting native fungal isolate. For this purpose 45 soil samples of 15 cherry orchards (each three samples) were collected and EPF were isolated using Galleria bait method. Results of this research revealed that 75.5% of soil samples had fungi. Occurrence of EPF in different orchards was not significantly different. Two fungal species were identified, *Beauveria bassiana* and *Metarhizium anisopliae*. Out of 45 soil samples, 55.5% had only *B. bassiana*, 6.67% had only *M. anisopliae* and 13.33% had both species. In preliminary tests, all fungal isolates were pathogenic to Cherry fruit fly larvae. The results suggest that cherry orchard soils in Shahrood district are rich of EPF and can be used for managing of this pest.

**Keywords**: Entomopathogenic fungi, *Rhagoletis cerasi*, microbial control


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**Erwinia aphidicola on Phaseolus vulgaris and Pisum sativum: a new pathogen in Spain**

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During 2003 and 2004, leaf spot disease of common bean (*Phaseolus vulgaris*) was observed in southeastern Spain (Almeria, Granada and Malaga) (Gonzalez et al. 2005) and symptoms of generalised chlorosis as well as necrosis in leaves and tendrils were observed in *Pisum sativum* cv. *Tiraque* (Gonzalez et al. 2007). In 2006 and 2007, samples of common bean (cv. Donna) with chlorotic and necrotic leaf spots were collected from Almeria to determine the pathogen. Bacteria isolated from leaves with spots exhibited the biochemical characteristics of the family Enterobacteriaceae. They were Gram-negative, oxidase negative, catalase positive, fermentative, rod shaped, motile, facultatively anaerobic. Two isolates were selected for pathogenicity tests. Bacterial suspensions (10^8 cfu/ml) were spray inoculated on bean seedlings (*Phaseolus vulgaris*) cv. Donna (2-3 true leaves). Beans were covered with transparent plastic bags for two days and held in an incubation chamber at 22 °C and 80% relative humidity with a 12 h photoperiod. Assays were conducted twice. Symptoms that developed were similar to those originally observed in the field. No symptoms were observed on control plants (inoculated with distilled water). Preliminary identification of the pathogenic isolates based on 16S rDNA sequencing was as either *Erwinia persicina* or *E. aphidicola* (99–100% homology). Primers for PCR amplification of partial sequences of *dnaJ*, *recA* and *gapDH* were manually designed from sequences of *Erwinia persicina* from GenBank (Accession Nos. AB272647, DQ859883 and AF165028, respectively). The amplified sequences were compared with available DNA sequences by using BLAST giving 100 % homology with *recA* and *gapDH*, and 99% with *dnaJ* of *E. aphidicola*. The same results were obtained for isolates from *Phaseolus vulgaris* (LPPA 373) and *Pisum sativum* (LPPA 408) obtained by González et al. (2005, 2007). Additional biochemical and pathogenicity tests and molecular analysis were performed using *E. persicina* ATCC 49742, *E. persicina* ATCC 35998 and *E. aphidicola* GTC 1688 as controls in which 91%, 85% and 88% homology with *recA*, *gapDH*, and *dnaJ* of *E. persicina* was found. Previously, *E. aphidicola* was isolated from the pea aphid, *Acyrthosiphon pisum* (Harada et al. 1997), so to our knowledge, this is the first report of *E. aphidicola* as a plant pathogen of *Phaseolus vulgaris* and *Pisum sativum*. Our results confirm that sequence analysis of 16S rDNA may not provide sufficient resolving power in discriminating closely related species.
Evaluation of flow cytometry to assess Erwinia amylovora viability under different stress conditions

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Erwinia amylovora is a plant pathogenic bacterium causing fire blight, a serious disease that affects cultivated and wild plants of the Rosaceae family worldwide, some of great economical importance as pear and apple trees. Fire blight is a difficult to control highly contagious disease, since the pathogen is easily spread and able to survive in many different reservoirs under adverse environmental conditions. Understanding the persistence of E. amylovora in these conditions would improve the control strategies against the disease but little is known on this topic up to now.

In the last years, it has been demonstrated that E. amylovora is able to survive in environmental oligotrophic water by adopting the starvation-survival state and the viable but non-culturable (VBNC) state, strategies of non-sporeulating bacteria against environmental stress to favour their survival. E. amylovora also becomes VBNC after exposure to some chemical compounds such as copper, which is frequently used for fire blight control. In that physiological state, bacterial cells are characterized by their inability to be cultured on conventional nonselective media while remaining viable as evidenced by culture-independent methods, such as fluorescence microscopy (FM) after viability staining. However, counting methods based on microscopy are time-consuming and influenced by investigator bias effect. Flow cytometry (FCM) has been reported as a faster and more accurate counting method than FM. Therefore, we have evaluated FCM to assess E. amylovora viability under different stress conditions by using Live/Dead staining combined with FCM detection. To validate the FCM results, FM was performed in parallel. Thus, E. amylovora suspensions at 10^7 cfu/ml were exposed to chlorine, acetic acid, hydrogen peroxide and copper at several concentrations. After each treatment, samples were taken for cultivable cell counts on King’s II medium and for viable and total counts after Live/Dead staining. Bacterial counts by FCM and FM were compared.

The results of this study have confirmed that E. amylovora becomes non-culturable on plates after exposure to different stressful treatments, varying the viable fraction of the population according to the type and concentration of the assayed compound. Interestingly, counts obtained by FCM agree well with those obtained by FM, although FCM often gave slightly higher counts than those obtained by FM. Moreover, FCM allowed a clearer and faster discrimination of E. amylovora subpopulations of viable and non-viable cells. Then, FCM is a rapid and reliable technique for quantification of E. amylovora viable populations that can be applied to study the physiological state of the pathogen under diverse adverse environmental conditions and may provide new knowledge about the epidemiology of this disease required to improve its control.

Keywords Plant pathogenic bacterium; fire blight; cytometry; fluorescence microscopy; chlorine; copper; acetic acid; hydrogen peroxide; VBNC.

Evaluation of plant growth promoting and colonization ability of bradyrhizobia isolated from sweet potato.

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Sweet potato (Ipomoea batatas L.) is an important root crop for staple food throughout tropical and warm temperate regions. The field study indicated that the sweet potato can be grown in a low fertile soil with little fertilizer application, and possible input of N2-derived nitrogen by endophytic nitrogen fixation has been suggested (Yoneyama et al., 1998). A study of the diversity of endophytic diazotrophs by culture-independent method revealed the presence of bradyrhizobia within the stems and storage roots of sweet potato (Terakado-Tonooka et al., 2008). We have isolated several diazotrophic bacteria from surface-sterilized sweet potato tissues, and identified Bradyrhizobium strains based on 16S rDNA and nifH sequence analysis. To assess the potential of Bradyrhizobium as a growth promoting, nitrogen fixing entophyte, two Bradyrhizobium strains, AT1 that has been isolated from storage roots of potatoes were inoculated to micropropagated sweet potato under axenic conditions. Increase in top fresh weight upon inoculation of either AT1 or MAFF210318 was recorded as compared to uninoculated plants after 55 days of growth. Internal populations in the stems of inoculated plants reached to approximately 10^7 to 10^8 cells per gram fresh weight at the end of the experiment, whereas no bacteria could be isolated from the uninoculated control plants. The analysis of nitrogen fixation using 15N-isotope dilution technique suggested some contribution of N2 fixation in plants, although high variability among replicates in inoculated plants was noted. These results suggest that bradyrhizobia isolated from sweet potatoes can colonize in the stems and promote the growth of sweet potato.

Keywords Bradyrhizobium; endophyte; nitrogen fixation; sweet potato

References:
Terakado-Tonooka J. et al. (2008) Expressed nifH genes of endophytic bacteria detected in field-grown sweet potatoes (Ipomoea batatas L.), Microbes Environ. 23, 89-93
Fiber degrading potential of rumen fungi isolated from cattle

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Since the time rumen fungi were discovered, they have been explored in many ways by the rumen microbiologists and animal nutritionist. Among the various accomplishments, their use in the development of direct-fed microbials is most significant. This approach of feeding rumen fungi as direct-fed microbials is gaining popularity as it improves the animal productivity by changing the rumen environment. Anaerobic fungi have ability to produce wide array of hydrolytic enzymes (such as cellulases, xylanases, esterases etc) which helps in degradation of lignified plant-cells walls. Fibre degradation is an important characteristic of rumen fungi because rhizoids of fungi penetrate plant tissue far better than bacteria and protozoa. In addition, they have been found to produce conjugated linoleic acid, which has many health promoting attributes.

In present study, rumen fungi were isolated from fistulated cattle using Hungate roll-tube technique. 20 fungal isolates were selected based on difference in their morphological features (viz., thallus morphology and rhizoid type). For assaying their enzymatic activities, these isolates were grown in Joflin’s broth supplemented with 0.5% of carboxymethyl cellulose (CMC), avicel, cellobiose and xylan separately, for assaying the activity of CMCase, avicelase, β-glucosidase and xylanase respectively. After 96 hours of incubation at 39°C, the cultures were centrifuged and culture supernatants (enzyme) were then analyzed for estimation of reducing sugars (i.e. glucose for CMCase and avicelase; xylose for Xylanase and p-nitrophenol for β-glucosidase) employing dinitrosalicylic acid method. For CMCase, β-glucosidase and xylanase activity the reaction mixture was comprised of 0.2 ml culture supernatant mixed with 1.8 ml of 100mM phosphate buffer (pH 6.5; 10 mg/ml of substrate concentration). The reaction mixture was then incubated at 50°C for 30 min for xylanase, CMCase and β-glucosidase and at 40°C for 4 hours. Absorbance was taken at 575 nm for CMCase, avicelase and xylanase and at 410 nm for β-glucosidase. The enzyme activities were calculated as IU, i.e., μmol of glucose or xylose or p-nitrophenol released per ml per min.

Among the 20 isolates, C-7 was found to have highest CMCase activity with 0.26 IU, while, avicelase activity (0.045 IU) was highest in C-15. Maximum xylanase activity (1.62 IU) was found with C-16 and C-10 found to have maximum β-glucosidase activity of 0.028 IU. The average activity of these isolates will also be assessed followed by molecular characterization, so that fungi possessing the maximum fibrolytic potential can be exploited for enhancing rumen productivity.

Fungal diversity associated to Prays oleae in Trás-os-Montes (Northeastern region of Portugal). A survey of potential entomopathogenic fungi

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2BioFG, Departamento de Biologia, Universidade do Minho, 4710-057, Braga, Portugal.

Olive groves are one of the main agricultural activities in the Portuguese region of Trás-os-Montes. They occupy a very large area, where new biological agricultural practices have been increasingly applied. In order to control the pests that attack this culture, using methods that do not rely on chemical substances, an effort must be conducted to achieve new processes. One of the most important pest in the olive groves in this region is the olive moth (Prays oleae Bern.), responsible for high losses in olive yields. One of the promising approaches to control this pest is the use of entomopathogenic fungi that naturally occur in the olive orchards and are able to infect and kill olive moths. The present work intends to evaluate the diversity of fungal species associated to P. oleae in several olive orchards located in Trás-os-Montes region. To achieve this goal, we collected larvae and pupae of the three annual generations (phytophagous, antophagus and carpophagus) of P. oleae. Whenever a fungal agent was associated to the cause of death of the moth, we proceeded to the in vitro isolation of the fungal specimen. Pure cultures of each fungus were obtained and were molecularly identified by sequencing the amplified internal transcribed spacer (ITS) region of rDNA. The higher diversity of fungal species was found in the phytophagous generation, followed by the carpophagus and antophagus. In the phytophagous generation, 77% of the total isolates were entomopathogenic fungi, whereas 18% were phytopathogenic fungi, being the remaining described as antagonistic fungi. Among the entomopathogenic fungi, the most frequent isolated species (97%) was the mitosporic ascomycete Beauveria bassiana. In the antophagus generation, 55% of the total isolates were phytopathogenic fungi, 41% were antagonistic fungi and only 4% corresponded to entomopathogenic fungi. In the carpophagus generation, more than 50% of the total isolates were phytopathogenic fungi. In this work, it will be discussed the extent of the fungal presence in P. oleae, as well as the characteristics of each fungal species and their applicability in pest control.

Keywords: Fungal diversity; Prays oleae; Entomopathogenic fungi; Beauveria bassiana
Fungal microbiota from rain water and pathogenicity of the isolated *Fusarium* species

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Using the dilution-plate method, 12 fungal genera were identified from rain water samples collected during January and February 2009 in Almería (Spain). The rain water analysis revealed a great content of airborne fungal spores (9.084 U.F.C. of *Botrytis cinerea* were recovered per m³). Some of them have been considered as plant pathogens, as for examples *Acremonium*, *B. cinerea* and the species of *Fusarium*. Others have been referred as cause of postharvest losses (*Aspergillus*, *Alternaria*, *Cladosporium*, *Fusarium*, *Penicillium* and *Rhizopus*). Some others have been used as biological agents to control pest in greenhouses (*Beauveria bassiana*).

Specific analysis of the *Fusarium* microbiota revealed the presence of three species of *Fusarium*: *F. oxysporum*, *F. proliferatum* and *F. equiseti*. Pathogenicity assays were conducted with 22 of the collected isolates of *Fusarium* species. Eleven isolates of *F. proliferatum*, six isolates of *F. equiseti* and five isolates of *F. oxysporum* were tested on tomato (*Lycopersicum esculentum* Mill.), melon (*Cucumis melo* L.), cucumber (*Cucumis sativus* L.) and pea (*Pisum sativum* L.) to evaluate their pathogenicity. Pre and Post-emergence pathogenicity was evaluated. The study of the pathogenicity showed that most of the isolates caused pre-emergence damping-off on tomato seedlings. In the case of melon, most of the isolates of *F. oxysporum* (six out of seven) caused pronounced damping-off in pre-emergence. *F. proliferatum* and *F. equiseti* did not show pathogenicity prior to emergence on cucumber and melon. Conversely, both *Fusarium* species caused root rot after emergence of cucumber and melon seedlings. Pea seedlings were affected by *F. equiseti*, *F. oxysporum* and *F. proliferatum* causing damping-off prior to emergence. Pathogenicity (root rot) was also observed after emergence.

This work will allow knowing some epidemiological aspects of plant pathogenic fungus in natural environments. The presence of pathogenic species within the *Fusarium* genus in rain water could indicate long distance dispersal in natural environments.

**Keywords** airborne mycoflora; aeromycobiota; *Fusarium oxysporum*; *Fusarium proliferatum*; *Fusarium equiseti*.

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Fungi and actinomycetes isolated from plant-parasitic nematode infested soils and their biocontrol potential, indole-3-acetic acid and siderophore production

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A total of 150 microbe isolates were obtained from 23 rhizospheric soil samples of chili, egg plant, guava, head lettuce, rice and fitweed infested with plant-parasitic nematodes. Sixty-seven isolates (44.67%) were fungi and 83 isolates (55.33%) were actinomycetes. The predominant fungal species were *Penicillium* sp. (32.8%), *Aspergillus* spp. (7.5%), *Cladosporium* spp. (6.0%) and *Paecilomyces* spp. (6.0%). The predominant actinomycete species were (97.6%) were predominant actinomycete species. All of fungal and actinomycete isolates were evaluated in vitro for their effects on egg hatch and juvenile mortality of *Meloidogyne incognita*. The Results showed that 10 isolates of fungi and 7 isolates of actinomycetes significantly reduced egg hatch rate and also killed hatched juveniles after 7 days. Four isolates of actinomycetes against both of *M. incognita* and fungal plant pathogens. Ten nematophagous microbe isolates showed the abilities to produced indole-3-acetic acid (IAA) and 9 isolates produced hydroxamate siderophore. *Streptomyces* spp. CMU-MB021 reduced egg hatch rate to 46.5% and increased juvenile mortality rate to 76.4% contrasted to the control of 79.6% and 3.6%, respectively. Furthermore, it had high ability to produced antifungal compounds, IAA and siderophore. This strain may be useful to control plant disease and promote plant growth in the agricultural fields in the future.

**Keywords** nematophagous fungi actinomycetes root-knot nematode antifungal Siderophore Indole-3-acetic acid
Phosphate-solubilizing rhizobacteria improve soil fertility by converting insoluble phosphorus forms to soluble forms accessible by plants. One hundred and three phosphate-solubilizing bacteria were isolated on modified Pikovskaya agar from the rhizosphere of pea cultivated at different locations in Lahaul and Spiti situated between 31 ° 45' 57" N and 72 ° 59' 57" N and 76 ° 46' 29" and 78 ° 41' 34" E in the Indian trans-Himalayas. Eighteen isolates producing prominent phosphate- solubilization zones were evaluated for quantitative estimation of inorganic phosphate solubilization, siderophore production, ACC deaminase activity, production of IAA-like auxins, and plant growth promoting activity in maize and pea under environment controlled conditions. The bacterial isolates significantly enhanced root length, shoot length, and dry weight over the untreated control in maize and pea.

Amplified ribosomal DNA restriction analysis of the rhizobacteria placed the bacteria under 14 groups, while 4 isolates stood independently outside the groups. The representative isolates of ARDRA groups were characterized using BI'OLOG, FAME analysis and 16S rDNA sequencing. The rhizobacterial strains showed identity with Bacillus cereus, B. megaterium, B. silvestris, Brevibacillus agri, Enterobacter cloaca, Myxococcus odorum, Ochrobactrum grignonense, Pantoea agglomerans, Pseudomonas chlororaphis, P. fluorescens, P. mendax, P. putida, P. syxanthaxa, Providencia rustigianii, Stenotrophomonas maltophilia, S. nitritireducens, S. rhizophila, and Varivoras paradoxus. Fluorescent Pseudomonas were found to be the dominant phosphate-solubilizing rhizobacteria in pea rhizosphere in the Lahaul and Spiti cold deserts of the Indian trans-Himalayas. Work has led to the selection of Pseudomonas putida strain BIHB 1369 and P. fluorescens strain BIHB 1433 representing the widespread genotypes with multiple plant growth promoting attributes. The strains could find use in developing microbial inoculants for widespread application.

Keywords plant growth promoting bacteria; diversity; widespread genotypes; Indian trans-Himalayas
Genetic Variability Analysis of entomopathogenic fungi isolated from citrus-growing areas of Mexico

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Mexican producers hold first place in the world as lemon producers of the variety “Mexican” and second as producers of lemon variety “Persian”. The states of Mexico who excel in citrus production are: Yucatan, Campeche, Tabasco, Veracruz, Tamaulipas, San Luis Potosi, Oaxaca, Michoacan, Colima, Sinaloa and Sonora. Mexico is the fifth largest producer of citrus in the world, covers an area of 520 hectares established in 23 states, occurring in approximately 6.7 million tons annually, with a value of over 8.950 million Mexican pesos. Currently, the citrus industry in Mexico is at risk caused by the Citrus Tristeza Virus where biological control of vector is essential to prevent the spread of this disease and more recently the threat of the plague known as Huanglongbing (HLB) or yellow dragon. It is considered that citrus regions are important for biological control with entomopathogenic fungi acclimatized to each region, so that these native agencies perform better. For these reasons, in this investigation native microorganisms citrus regions of Mexico were isolated and characterized genetically, with the aim of establishing a collection of entomopathogenic fungi, so that in the future it could have the potential to solve regional problems involving citrus pests. We collected 142 soil samples from citrus areas of Mexico: Nuevo León (23), Sinaloa (17), Yucatán (5), Campeche (13), Tabasco (5), Tamaulipas (20), San Luis Potosi (25), Michoacán (15) and Sonora (17). We used larvae of Galleria mellonella as bait to trap, multiplicate and detect entomopathogenic fungi in vivo. The isolated fungi were purified and kept in vials with glycerol at - 80 °C, subsequently conducted the microscopic and molecular identification. Of all the soil samples processed 23% were positive in the presence of entomopathogenic fungi according to the macroscopic and microscopic characteristics: Beauveria was detected in 12% (17 isolates), Metharizium in 1% (2 isolates) and Paecilomyces in 10% (14 isolates). Beauveria was detected in Sinaloa, Tamaulipas, Nuevo León and San Luis Potosi (9, 4, 3 and 1 isolates, respectively); Paecilomyces in Nuevo Leon, Tamaulipas, San Luis Potosi, Sonora, Campeche and Tabasco (8, 2, 1, 1, 1, and 1 isolates, respectively) and only Metharizium in Nuevo Leon (2 isolates). We analyzed the genetic variability by using the sequences of internal transcribed ribosomal genes (ITS) and subsequently performed the digestion with the enzyme Hae III. The results of the PCR generated fragments of approximately 600 bp for genus Beauveria spp, and Metharizium spp. In the case of Paecilomyces spp it amplified a fragment of 650 bp. The restriction analysis of these fragments generated for Paecilomyces spp. 4 fragments of 257, 131, 89 and 56 bp, for Metharizium spp., were obtained fragments of 404 and 141 bp and for the case of Beauveria spp., 287, 119, 89, 56 and 19 bp. Both reference strains and field isolates obtained similar restriction patterns. Genera of native entomopathogenic fungi were found on each state; this opens the field inside the biological control of pests affecting citrus in each region.

Keywords: entomopathogenic fungi, PCR-RFLP, citrus

Genetic variation within AMF morphotypes from mycorrhizosphere of plants from undisturbed, industrial and agricultural land: An investigation through LSU rDNA sequencing

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Arbuscular mycorrhiza fungus (AMF) communities of field soil collected from undisturbed, industrial and agricultural land were surveyed by establishing their trap cultures. Spores of AMF were collected from these trap cultures by wet sieving and their morphology was observed, based on which they were grouped into different morphotypes. DNA was isolated from a single spore and partial sequence analysis was conducted using 28S rRNA gene. Nested polymerase chain reaction (PCR) with Glomus specific primer was used to identify species. Topologies obtained by using extracted LSU rDNA sequences revealed three phylogenetic clusters within the clade of Glomus group A. Phylogenetic analysis also revealed the presence of different sequences within clones of a single spore isolate. In addition, sequences obtained from two different isolates originating from the same field clearly fell in separate clusters of Glomus. The combined analysis of spore morphology and LSU rRNA D1-D2 region sequences obtained from this study showed that there are some isolates similar in morphology but which did not group together within the same cluster of Glomus. Present study revealed higher population of Glomus intraradices in agricultural soil as compared to the industrial wasteland and uncultivated soil. In contrast, loss of non-Glomus AMF in agricultural soil was observed. The difference is due to complex selection pressure, which increases the population of Glomus intraradices in agricultural soil. PCR probes were designed from D1-D2 region of LSU rRNA gene to monitor the presence of selected AMF after application into the field. Our data indicates that use of LSU rRNA sequences is a suitable tool to investigate Glomeromycota diversity in the soil of different ecological habitats.

Key words – Arbuscular mycorrhiza fungi; Mycorrhizosphere; Industrial wasteland
Glomalin production and microbial activity in soils impacted by gypsum mining in the semi arid of Pernambuco

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In order to evaluate and compare the biological state of soils in a preserved, native, “caatinga” area and impacted gypsum mining areas. Soil samples were collected in four areas: a native, preserved area “caatinga”; surroundings of the mine; waste deposit area; interface between the waste deposit area and a degraded “caatinga” area. The values of hydrolysis of FDA, C from microbial biomass, basal respiration were higher in the preserved “caatinga” area than in the impacted areas. The gypsum mining activity reduced the concentration of easily extractable glomalin in relation to the one found in the native “caatinga” area in both collecting periods. Higher deposits of total glomalina also occurred in the native area, mainly during the rainy period. The mining activity produced a negative impact in the soil macrobiotic, reducing the total enzymatic activity. The microbial biomass was significantly lower in the waste deposit area than in the native and interface areas. The results indicated that the mining activity is harmful to the soil microbiota of the area and that glomalina can be a useful indicator of soil disturbance.

Keywords: Glycoprotein; microbial biomass; gypsum activity; AMF.

Host specificity and pathogenic ability of Phytophthora parasitica and P. capsici on tomato and sweet pepper.

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Nineteen wild strains of P. parasitica and six of P. capsici, isolated from diseased tomatoes and sweet peppers respectively, and 8 P. parasitica strains from natural infested sweet pepper soils, were all inoculated on tomato and sweet pepper simultaneously to study host specificity. Two inoculation methods were used: a) irrigating with a Mal-extract agar (MEA) fungal suspension the sterile vermiculite-substrate containing the plants, b) cutting the shoot of the plants and putting a 1cm-diameter disc of MEA containing mycelium and sporangia. So there were 4 treatments per isolate: tomato+drenching, tomato+agar disc, sweet pepper+drenching, sweet pepper+agar disc. Three replicates were incorporated per treatment and isolate. Each replicate consisted on five plants in a 1L plastic pot that were inoculated by drenching when showed two to three true-leaves, and were inoculated with the agar disc when showed four to five true-leaves. Plants inoculated by irrigation were observed for wilting for 30 days. For plants inoculated with the agar disc, root tissue advance was measured during 12 days, where appeared. All trials were carried out in a climatic chamber with 23-27 ºC and 16:8 (light:dark). Controls consisted on three replicates per vegetal species irrigated with a suspension of MEA and other three replicates per species whit cut shoots and a disc of MEA placed on. Isolate identification was achieved through morphological taxonomy and confirmed by sequencing of amplicons of the internal transcribed spacer region (ITS) rDNA. Results highlight the differential response of the isolates. All P. capsici isolates were highly pathogenic to both species by both drenching and cut short inoculation, showing no host specificity. From the 19 P. parasitica strains originally isolated from diseased tomato plants, only 3 were pathogenic to sweet pepper, but 11 were to tomato when irrigated with the fungi. When these isolates where cut shoot inoculated only 3 showed stem rot on sweet pepper, but 10 did it on tomato. About the P. parasitica isolates obtained from soils, only two were pathogenic on tomato and three on sweet pepper, in all cases showing low pathogenicity on the stems. These results reflect the host specificity of P. parasitica when comes from root and crown rot diseased tomato plants. This specificity doesn’t exist for P. capsici isolates coming from blighted sweet pepper plants.

Keywords: Solanum lycopersicum; Capsicum annuum; P. nicotianae; parasitism; blight; root rot; crown rot.
Identification of virulence genes in *Fusarium oxysporum* f. sp. *lycopersici* the causal agent of tomato wilt disease

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*Fusarium oxysporum* f. *sp. lycopersici* is a soil-borne fungus that causes wilting disease in tomato. The soilborne *Fusarium oxysporum* strains, plays a significant role in disease development. The abilities of *F. oxysporum* f. *sp. lycopersici* isolates G010; 649-91; 160-57 to produce FA in infected *Gladiolus* corm tissues was evaluated, in relation to the presence of two biological control agents (BCAs), *Trichoderma harzianum* T22, and *Aneurinobacillus migulanus*. Tests of pathogenicity were used to differentiate between the ability of *Fusarium oxysporum* strains to secrete FA. FA was identified using LC/MS and quantified using HPLC. G010 was the only isolate that produced FA among the three *Fusarium* isolates. The mycelium was ground in liquid nitrogen using a pestle and mortar and the Genomic DNA extraction was performed using the Qiagen Genomic DNA purification kit (Qiagen, Germany) following manufacturer's instructions. The identification of *SIX* genes was performed using primers SIX1, SIX2, SIX3 and SBH1. PCR with the SIX1, SIX2, SIX3 and SBH1 primers set amplified a 647-bp fragment from three races of the *Fusarium oxysporum* f. *sp. lycopersici* (FOL). Sequence analysis was performed on an ABI 3700 DNA Analyzer. Raw sequences from both strands were aligned and edited visually using BioEdit. Alignments of sequences were also examined using Clustal X Multiple Sequence Alignment Program version 1.81. The sequences obtained were compared with the *Six* genes sequences available from GenBank using a BLASTn search. The three genes were grouped in one clade where races 1 and 2 showed a genetic identity of 100% and a similarity of 98%, while race 3, the similarity was lower (85%). For *sixa2* and *sixa3 sbh1* genes, the race 3 showed a very different behavior from races 1 and 2. Where identity (56%, 50%, 50%) and similarity (65%, 65%, 60%) were much lower than in races 1 and 2, respectively. Our findings have practical implications for the detection and identification of *F. lycopersici*. These genes may be part of a larger, dispensable region of the genome that confers the ability to cause tomato wilt and has spread among clonal lines of *F. oxysporum* through horizontal gene transfer. Identification of genomic regions contributing to the distinction of races when combined with other markers and may help the development of molecular markers race-specific to be used in the characterization of isolates of *F. oxysporum* f. *sp. lycopersici* circulating in different counties tomato growers of the state of Pernambuco and Brazil.

Keywords: *Fusarium oxysporum*; disease resistance; xylem; tomato.

Impact of Biological Control Agents on Fusaric acid concentrations in *Gladiolus grandiflorus* corms Infected with *Fusarium oxysporum* f. sp. *gladioli*

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Fusaric acid (FA) (5-n-butylpuridine 2-carboxyl acid), a highly toxic secondary metabolite produced by strains *Fusarium oxysporum* strains, plays a significant role in disease development. The abilities of *F. oxysporum* f. *sp. gladioli* isolates G010; 649-91; 160-57 to produce FA in infected Gladiolus corm tissues was evaluated, in relation to the presence of two biological control agents (BCAs), *Trichoderma harzianum* T22, and *Aneurinobacillus migulanus*. Tests of pathogenicity were used to differentiate between the ability of *F. oxysporum* strains to secrete FA. FA was identified using LC/MS and quantified using HPLC. G010 was the only isolate that produced FA among the three examined, and was the most virulent isolate on gladiolus. The presence of *T. harzianum* prevented FA secretion into the corms. In the presence of *A. migulanus*, however, the amount of FA secreted in to the corm tissues increased. These results support the use of *T. harzianum* as an effective biological control agent against *F. oxysporum* f. *sp. gladioli*.

Keywords: Fusaric acid; *Trichoderma harzianum*; *Aneurinobacillus migulanus*; Gladiolus
**In vitro** assessment of fungal endophytes’ ability to confer drought and heat tolerance to wheat

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Fungal endophytes, which can be defined as fungi living asymptomatically within plant tissues, can benefit host plants in a variety of ways, including enhanced tolerance for abiotic or environmental stresses. This presentation aims to determine if one or more fungal endophytes can enhance drought or heat tolerance in wheat-host through the relationship between fungi and seeds in co-culture, known as “mycovitality”, and to investigate the ability of the same fungal endophytes to tolerate drought as free-living organisms **in vitro**. Drought tolerance was assessed **in vitro** using potato dextrose agar (PDA) media with 8% polyethylene glycol (PEG) to simulate drought stress. Heat stress was induced in an incubator, held at 36 °C. Wheat stress tolerance was measured in terms of percent seed germination at 3 days and seedling fresh weight at 7 days. The stress tolerance of free-living fungal organisms was measured in terms of survival and colony growth rate. Three of the 6 fungal endophytes stressed showed potential to improve wheat tolerance for heat or drought **in vitro**.

**Keywords:** Fungal endophytes, abiotic stress, wheat

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**Indole-3-acetic acid production by plant associated bacteria: potential to alter endogenous IAA content and growth of *Triticum aestivum* L.**

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Bacterial strains of *Bacillus, Pseudomonas, Escherichia, Micrococcus* and *Staphylococcus* genera isolated from rhizosphere, histoplane and phylloplane of different plant species were identified by 16S rDNA gene sequencing. Strains were evaluated to enhance endogenous indole-3-acetic acid (IAA) content and growth of *Triticum aestivum* var. Inqalab-91. Gas chromatography and mass spectrometric (GC-MS) analysis revealed that bacterial strains produced 0.6 to 8.22 μg IAA ml⁻¹ in the presence of precursor L-tryptophan. Plant microbe experiments showed a significant positive correlation between auxin production by bacterial strains and endogenous IAA content of *T. aestivum* (r = 0.618*) and colorimetric analysis (r = 0.693**). Similarly, highly significant positive correlation for shoot length (r = 0.627**) and shoot fresh weight (r = 0.620**) was observed with auxin production under axenic conditions. Bacterial inoculations also enhanced shoot length (up to 29.16%), number of tillers (up to 97.35%), spike length (up to 25.20%) and seed weight (up to 13.70%) at final harvest in wire house experiments. In the end, it can be concluded that bacterial strains have the ability to increase the endogenous IAA content and growth of *T. aestivum* var. Inqalab-91. Hence, microbial strains associated with different plant species can be effectively used to enhance the growth and yield of agronomically important crops.
Influence of organic and conventional soil tillage system on soil respiration and enzymatic activity.

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Soil enzyme activities can act as an indicator of potential microbial activity and often correlate with other indicators of activity such as soil respiration, and microbial biomass. Soil enzyme activities may also provide some insight into the metabolism of the soil. Measurements of soil respiration and enzyme activities can related to soil microbiological activities and thus, may be used as an index of soil functioning. Soil microbial and enzymatic activity depends on tillage system, cultivated plants and environmental conditions.

The aim of experiment was to study the influence of tillage on selected soil microbiological properties. Soils were sampled from nine plots with two variants of tillage methods: conventional (six plots) and organic (three plots). Soils samples were collected five times during vegetation period. Soil basal and induced respiration, activity of dehydrogenase, urease and fluorescein diacetate (FDA) hydrolytic activity was detected.

The results indicate different soil tillage system and crop rotation influence on soil biological activity. The activity of dehydrogenase showed clear gradient among systems and sampling time in comparison with urease and FDA.

The highest microbial biomass (C-biomass) was detected in the conventional oats field at the beginning of vegetation period.

Keywords: soil respiration, dehydrogenase, urease, FDA

Influence of the C:N ratio and pH on ectomycorrhizal fungal growth

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Wild edible mushrooms are a natural resource with a high nutritional and economic value. Several studies have demonstrated that the commercially mushroom harvesting could be an important additional source of income in both developing and developed countries. The species most appreciate in the entire world are ectomycorrhizal (EM) and are usually the most valued. This aspect derived essentially from the difficulty to obtaining these symbiotic fungal species by culture. The main objective of the present study is to assess the influence of the C:N ratio and pH on the growth of three EM fungal species, under in vitro conditions. The species studied were Lactarius deliciosus Fr., Suillus bovinus L.:Fr. and Boletus edulis Bull.:Fr, all with high economic importance. The fungal were cultivated on solid modified Melin-Norkrans (MMN) medium with different C:N ratio (37.7, 75.5, 113.2) at pH 4, 5, 6 and 7, and fungi growth was determined over 63 days of culture. Morphological characteristics of each fungal culture were also assessed. The results obtained showed that both C:N ratio and pH had influence in fungal growth. The growth of B. edulis and L. deliciosus were significantly higher in medium with 113.2 C:N ratio when compared to other C:N ratios. By contrast, S. bovinus have the highest growth on medium with a C:N equal 75.5. The best pH value for S. bovinus, B. edulis and L. deliciosus growth was, respectively, 5, 6 and 4. No macroscopic nor microscopic alterations in mycelial morphological characteristics between isolates growth at different C:N ratio and pH were detected.

Keywords: Suillus bovinus, Boletus edulis, Lactarius deliciosus, ectomycorrhizal, C:N, pH, culture medium.
Influence of the non-symbiotic soil basidiomycete, *Stropharia rugoso-annulata*, on enzymatic activities in tissues of white mustard plants under natural conditions

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Although the presence of herbaceous plants is crucial to the development of certain non-symbiotic soil basidiomycete fungi, the nature of their interactions receives little attention. This applies in particular to the release of fungal enzymes in rhizospheres of herbs but also to fungal influence on the enzymatic status of the plant itself. Under gnotobiotic conditions, fungal production of several hydrolytic and oxidative enzymes involved in lignocellulose and xenobiotic degradation increases during mycelial contact with roots of white mustard (*Sinapis alba* L., unpublished). In similar interactions of plants with soil bacteria, induction of bacterial dioxygenases is presumably stimulated by root exudates such as terpenes and phenols and results in an accelerated degradation of PAHs and PCBs [1-2]. In the present study, pre-germinated seeds of white mustard were transferred to triplicate 200-ml samples of untreated arable soil (pH4.5, C total, 7.20 %) densely permeated by the mycelium of the soil basidiomycete, *Stropharia rugoso-annulata* (*Stru*). Planted non-inoculated and unplanted/inoculated soil samples served as controls. The plants were harvested at the second-true leaf state (75 to 110 mm long). Shoot and root homogenates and soil extracts were examined for activities of enzymes which are involved in the degradation of xenobiotics and/or in the control of plant stress. It was the goal to record changes in plant and fungal enzyme activities in pairings of a non-symbiotic and non-pathogenic fungus with an herbaceous plant under widely natural conditions. Relative to plants from non-inoculated soil, the presence of *Stru* increased peroxidative. Mn-dependent peroxidase, and Remazol BBR esterase activities in shoot tissue of mustard to the 8-;>35-; and 130-fold, respectively, whereas the oxidative activity did not significantly change. Surprisingly, the corresponding activities in root tissue diminished to the 0.3-; 0.6-; and 0.2-fold and increased in regard of the oxidative activity to the 5-fold due to fungal influence. Unlike the conditions under gnotobiosis, the presence of the plant did not significantly stimulate the oxidative activity of *Stru* in the rooting soil where the fungus reduced the concentration of pant-released peroxidase to 17 %.

It is concluded that the strongly increased oxidative activity in roots of white mustard was caused by uptake of fungal laccase. This was confirmed in electrophoretic comparisons of fungal culture fluids and root extracts. Shoot oxidative activities were apparently pretend by the interaction of traces in active oxygen species with plant peroxidase. Whereas the strong increases in shoot peroxidase are typical of plant responses to stress [3], the loss in root peroxidase, an enzyme mainly located on the root surface, must be attributed to fungal proteolytic activity. It is further concluded that the rates of Remazol BBR decolorization and the formation of Mn⁺⁺⁺ are closely correlated with the activity of plant peroxidase (r = 0.999) which is able to form this abiotic oxidant upon the reaction with plant phenolics [4-5]. The presence of a Mn-peroxidase variant in plants is not postulated. The nature of fungal metabolites with the potential of exerting stress to herbs is discussed.

**Keywords** Mn⁺⁺⁺; oxidase; peroxidase; Remazol Brilliant Blue-R; soil basidiomycete; white mustard

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Isolation and comparative molecular diversity analysis of fluorescent pseudomonads by using four DNA fingerprinting techniques

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Fluorescent pseudomonads from chickpea, maize and jatropha rhizospheric soil were isolated and screened for the production of enzymes and hormones such as indole-3-acetic acid (IAA), hydrogen cyanide (HCN), ammonia, 1-aminocyclopropane-1-carboxylate (ACC) de-aminase, phosphorous solubilization and antifungal assay. Out of 34 isolates, 24 isolates produced plant growth hormone IAA in the presence of Tryptophan, 10 isolates produced ACC deaminase and fourteen of these isolates were the best in producing siderophore and indole acetic acid (IAA). In addition to IAA and siderophore-producing attributes, 33 isolates could solubilize phosphorus to various extents, 26 isolates were positive against three major phytopathogenic fungi viz. *Fusarium oxysporum* f. sp. *ciceri*, *Macrophomina phaseolina* and *Rhizoctonia solani*. Genotypic analysis was carried out using four different fingerprinting methods to assess their usefulness as tools to study the bacterial diversity within this complex group. The methods used were random amplified polymorphic DNA (RAPD), Amplified Ribosomal DNA Restriction Analysis (ARDRA) and repetitive element sequence-based PCR (rep-PCR) utilizing BOX and enterobacterial repetitive intergenic consensus (ERIC). The present study reveals the comparison among the various molecular methods for three different plants. Cluster analysis of the results clearly demonstrated the considerable heterogeneity among the isolates of different crops but homogeneity in the isolates of the same crop validating the above mentioned methods for studying intraspecific variation among the fluorescent pseudomonads. The knowledge on genetic diversity of fluorescent pseudomonads associated with chickpea, maize and jatropha rhizosphere is useful to understand their ecological role.

**Keywords** Fluorescent pseudomonads, 16S rDNA, ERIC, rep-PCR, Molecular diversity, DNA fingerprinting.

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References


Leaching of pathogens from manure to drainage water – assayed using classic and DNA/mRNA based methods

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The usual practice of addition of animal manure to soil can provide opportunity for contamination of soil and drainage waters. In a large multidisciplinary project involving many institutions the dispersion of different pathogens in agricultural soils has been assayed. In this study, we examined different pathogens using different techniques to evaluate the survival of the organism in soils: Plate/plaque counting, direct quantification of the organism in soils, and DNA-based qPCR.

In one experiment of the survival of Salmonella spp., three different factors were tested: temperature, soil type and manure treatment. A tetracycline-resistant Salmonella typhimurium culture was inoculated to yield 10^8 cfu/g into agricultural topsoil (with or without applied manure) or soil from the B horizon (below plough layer). Soils were stored at 5, 15 and 25°C simulating seasonal temperature exposure. The survival of Salmonella spp. assayed by plating techniques showed a superior survival at low temperatures, but a general decay was found in all samples. A high number of protozoa was found in the manured amended soil corresponding to a fast decay of inoculated Salmonella spp. Quantification of mRNA and DNA directly in the soil and manure samples showed that mRNA was degraded fast in soils at high temperature while mRNA was more stable at 5°C.

In a field experiment strings of manure were added into agricultural soil. During a period of two months, the sections of soils with different distance to the manure string were assayed to obtain information on survival and spread of E. coli (bacteriophage), faecal indicators (Enterococci, Bacteroides; E. coli) and tetracycline-resistant bacteria. The die-off of the different organisms was quantified showing an extended survival close to the manure-string. Genomic DNA from 400 tetracycline-resistant bacteria was isolated and their phylogenetic relationship was established using 16SrRNA gene sequencing showing that the main tetracycline-resistant bacterial species is E. coli.

Drainage water from the field was collected weekly from spring 2008. During the samplings in 2008, no tetracycline-resistant bacteria were found, but after manure applications in the autumn 2009 tetracycline-resistant bacteria were recovered. Again, a suite of different organisms were quantified, and in the first drainage water sample after manure application we found approximately 100 tetracycline-resistant cfu ml^-1. The total number of tetracycline-resistant bacteria in the manure was 1x10^5 cfu ml^-1.

In conclusion, the survival and environmental spread of pathogens and indicator organisms shows that not only the upper soil are impacted by the microorganisms originating from non-processed manure, but also drainage water can contain quite high numbers of the organisms. The results also show that DNA-based quantification of Salmonella spp. yields higher numbers than quantification based on mRNA indicating that mRNA will form a very conservative choice for pathogen quantification in environmental samples.

Keywords: DNA/mRNA extraction, invA genes; pathogens; public health; water quality; fecal indicator

Microbial analysis of soils from avocado crop modified by organic amendments

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One of the most important objectives of any sustainable system of agriculture is to maintain and improve the stock of soil organic matter adding organic amendments, like yardwaste mulches, manures and compost. This agricultural practice also has a direct impact on plant health and crop productivity. In Spain, the ecological production of avocado (Persea americana Mill.) is in extension due to the facilities of this crop for the ecological management, and the addition of organic amendments or mulches is one of the most popular actions performed by farmers. If adequate quality is provided, organic residues act not only as a source of nutrients for the plants, but also may increase size, biodiversity and activities of the microbial populations in soil.

The aim of this study is to characterize and compare different types of organic amendments applied to avocado soils. For this, a greenhouse experiment was performed using two years-old avocado plants growing on pots with soil supplemented with organic amendments. The different amendments were let mature simultaneously to the plant growth. After one year of maturation, the microbial diversity and enzymatic activities of soil and rhizosphere were analyzed. Microbial diversity was studied by culture-dependent and independent methods.

Microbial counts in selective culture media showed higher microbial populations in soils and rhizospheres of amended treatments if compare with control not amended treatment. The counts were especially high in amendments mixed with chicken manure and the composted grass. Aerobic sporulating bacteria and the Pseudomonas group were the most affected by the treatments. The DGGE patterns were more complex for the amended treatments, indicating higher microbial diversity in these soils. A deeper analysis of these patterns is still in progress, including quantification of some interesting differential bands. Enzymatic activities in soil and rhizosphere were determined by the API-ZYM system, able to analyze the presence and relative activity of 19 hydrolytic enzymes. Results showed higher activity of some of the analyzed enzymes in amended soil and rhizosphere if compare with control treatment.

Keywords: DNA/mRNA extraction, invA genes; pathogens; public health; water quality; fecal indicator
Microbial characterization of a heavy metal polluted soil phytoremediated with *Populus euroamericana*

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Heavy metal pollution is one of the major problems negatively affecting both human and environmental health and several technologies and methods have been developed to remove them from polluted soils. Traditional methods such as soil removal or extraction through chemical or physical means are costly from both an economic and an environmental point of view, and could potentially have a deleterious impact on soil physical, chemical, and biological properties.

Phytoremediation, consisting in the use of plants to remediate heavy metal polluted soils, is a clean and cost-effective technology that is likely to be readily accepted by a concerned public.

Metal phytoextraction can be influenced affected by microorganisms living on the surface or inside the plant roots and in the bulk soil. These rhizospheric microorganisms can act on pollutants, using their own degradative capabilities, but also positively affect plants by improving growth and health, enhancing root development, or increasing plant tolerance to various environmental stresses.

Clones of *Populus x euroamericana* I-214 were grown in an area, polluted by copper and zinc, close to an industrial site. In spite of similar chemical and physical features of the soil, the clones showed very different growth. In order to assess the possible role of the microflora in these heterogeneous plant growth, the microbiological properties of soil near roots of poplar with large (I-214G) or small (I-214P) size, as well as of the bulk soil (Soil), were characterized by coupling traditional culture-dependent (bacterial density, culturable bacteria identification, carbon utilization pattern and enzymatic activities) and -independent (Denaturing Gradient Gel Electrophoresis - DGGE) techniques.

The rhizospheric soils showed the highest microbial activity. Among culturable bacteria, Gram positive were predominant in the bulk soil, while *Variovorax* sp. was found only in rhizospheric soils. Since *Variovorax* sp. resulted to be specifically selected by the plant, six strains belonging to this specie, were characterized for their physiological traits possibly involved in plant growth promotion. Thus, enzymatic activities, production of auxin, salicylic acid , siderophore, L-aminocyclopropane-1-carboxylate (ACC) deaminase activity, as well as copper and zinc resistance were evaluated. DGGE analysis of Eubacterial and culturable bacteria communities showed low similarity among the sites. The culturable fraction and the whole Eubacterial community clustered separately. In addition, the cultivable fraction and the whole Eubacteria community of the bulk soil showed the lowest biodiversity. Band clonings and sequencing are in progress.

**Keywords** polluted soil; metabolic profile; enzymatic activities; microbial community structure; DGGE; rhizobacteria

Microbiological and chemical properties of Tarhana during fermentation

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Tarhana is a traditional Turkish fermented cereal food produced both commercially and in homes. Turkish fermented food made with cereal, milk products, various vegetables and spices using yoghurt bacteria and baker’s yeast as culture. Tarhana dough is prepared followed with lactic and alcoholic fermentation for 1–7 days. After fermentation the mixture is sun dried and ground. Similar products are known as “trahana” in Greece, “kisvak” in Egypt, “kustah” in Iraq and “tahana/talkuna” in Hungary and Finland.

White wheat flour, concentrated full fat yoghurt, tomato paste, onion, red and green paprika, mint and salt were used in tarhana preparation. Change in some chemical and microbiological properties of Tarhana in fermentation stage of production was investigated. During the 7-day tarhana fermentation period, acidity increased from 12.17 % to 36.11 %, pH decreased from 5.22 to 4.13, wet decreased from 70.12 % to 26.15 %. Average chemical composition of the Tarhana, at the end of fermentation, was wet 9.55 %, protein 20.05 %, total ash 5.65 % and salt 5.65%, fat 4.88 %.

During fermentation, count of lactic acid bacteria increased from 1.32 X 10$^6$ to 4.2 X 10$^7$ cfu/g and count of Total mesophilic aerobe bacteria increased from 1.75 X 10$^7$ to 2.88 X 10$^7$ cfu/g, count of yeast increased from 3.45 X 10$^4$ to 2.40 X 10$^5$ cfu/g, count of mould 1.55 X 10$^1$ to 2.45 X 10$^7$ cfu/g, content of Tarhana dough. Fermentation in Lactobacillus lactis spp. lactis, Leucostoc mesenteroides, Lactococcus acidophilus, Enterococcus durans, Pediococcus spp., Lactobacillus delbrueckii spp. lactis and Lactobacillus paracasei ‘s was seen to play a role. *Kluyveromyces marxianus, Yarrowia lipolytica,Pichia membranefaciens, Pichia mexicana, Pichia angusta, Debaryomyces hansenii A Candida sorbobulesa, Candida fluvatilis, Saccharomyces cerevisiae B* clark tanmlanmıştır. Fermentasyon sıresine *Kluyveromyces marxianus* ortamda kalınıtır.

**Keywords** pollute; metabolic profile; enzymatic activities; microbial community structure; DGGE; rhizobacteria

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Modeling of nitrogen leaching by using urea fertilizer in sandy loam soil

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To study of the N leaching process in sandy loam soil under drip irrigation system a full drip system was designed in Agricultural Research Farm, Shahrood, Iran. Selected crop planted for study was radish. In this experiment the lateral lines were spaced at 50 cm interval. The lateral lines were laid in such a manner that the same lateral line supplied water and fertilizer to all the randomized replicated plots. Plant to plant and row to row spacing were 10 cm and 30 cm, respectively. The recommended dose of fertilizers were 120 kg/ha of N, 50 kg/ha of P and 70 kg/ha of K. In this study urea was main source of applied nitrogen. Amount of water applied during each irrigation varied with water requirement. Amount of nitrogen applied varied according to the crop requirement and fertigation schedule. Modeling of nitrogen leaching blow the crop root zone was done by Hydrus-2D model. Amount of N going below the root zone depth was obtained by this model. Water and nitrogen patterns in the entire field described by analyzing the flow in the single volume element irrigated by single emitter. Simulation of nitrogen leaching was done in three types of soils. Results revealed that N leaching was highest in case of sandy loam soil and negligible in case of silty clay loam soil. This implies that in case of permeable soils like sandy loam, fertigation strategies play role in N leaching...
Molecular characterization of *Fusarium oxysporum* f. sp. *lycopersici* causing wilt of tomato

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*Fusarium* wilt of tomato (*Lycopersicon esculentum* Mill.), caused by *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) W.C. Snyder and H.N. Hansen, is an economically important disease and it is a destructive disease of tomato crop worldwide. With the coming of the molecular methods based on the analysis of DNA, these have been very useful tools in the phylogeny studies of *Fusarium* and in the differentiation of species, *formae specialis*, races and isolates. Three different host-specific races of pathogen (race 1, 2 and 3) have been identified. Three of the F. *sp. lycopersici*, f. sp. were assessed for genetic diversity using Random amplified polymorphic DNA (RAPD). Amplified Ribosomal DNA Restriction Analysis (ARDRA) was used to analyze the ITS1 – 5.8S rDNA – ITS2 region, amplified with *primers* ITS1 and ITS4. The genetic relationship for RAPD of the *F. oxysporum* races were analyzed using Unweighted Pair-Group Method with Arithmetic Averages (UPGMA) cluster analysis based on Simple Matching Similarity Coefficient. The amplified products were digested with the restriction enzymes *Abu* II, *Ban* II, *Eco* RV, *Hae* III, *Hinf* I, *Hpy* II, *Hpa* I and *Rsa* I and RAPD-PCR showed low variation among the *F. oxysporum* races studied, indicating close relationship among the races. Banding patterns generated for the enzymes it demonstrated a pattern monomorphic for the three races. The ARDRA technique, using these enzymes is not a promising marker to differentiate the *formae specialis lycopersici* from within the *F. oxysporum* complex. *Primers* ITS1/ITS4 for the region IGS had to the same behavior with relationship it analyzes of nucleotides. Where the races 1 and 2 formed an independent cluster and the race 3 showed a behavior paraphyletic in relationship the others. Cluster analysis of the combined data also showed that the *F. oxysporum* races were grouped into two main clusters with similarity value of 69% (races 1 and 2) and 63% (race 3) RAPD-PCR analysis, respectively. For the regions ITS and IGS the races of *Fusarium* had to the same behavior with relationship it analyzes of nucleotides. The regions ITS and IGS the races of *Fusarium* had to the same behavior with relationship it analyzes of nucleotides. Where the races 1 and 2 formed an independent cluster and the race 3 showed a behavior paraphyletic in relationship the others. Cluster analysis of the combined data also showed that the *F. oxysporum* races were grouped into two clusters, sharing 80 and 70% of genetic identity for the regions IGS and ITS, respectively for the races 1 and 2. However the race 3 showed an inferior genetic identity for 50% in relationship the two studied races. The results of the present study indicate that the *F. oxysporum* races were closely related.

**Key words:** ITS, IGS, RAPD-PCR, *Fusarium oxysporum* f. sp *lycopersici*

**Reference**


Molecular identification of endophytic actinomycetes isolated from *Aquilaria crassna* Pierre ex Lee and their plant growth promoters substances

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A total of 10 endophytic actinomycete strains were successfully isolated from healthy shoots and roots of *Aquilaria crassna* (Pierre ex Lec) and their plant growth promoters substances. Nonomuraea (1 isolate), *Actinomadura* (1 isolate), *Pseudococcidioides* (1 isolate) and *Nocardioides* (3 isolates). The remaining 2 isolates were not identified. All of isolates produced the amount of indole-3-acetic acid (IAA) and ammonia ranging between 9.85±0.31 to 15.14±0.22 μg/ml and 2 to 60 mg/ml, respectively. Among 10 isolates tested, the amount of hydroxamate-type siderophore produced by 2 isolates was undetectable. While the remaining 8 isolates produced the amount of hydroxamate-type ranging between 3.21±0.12 to 39.30±0.40 μg/ml. Also, catechols- type siderophore produced by 9 isolates was undetectable. *Actinomadura glaucoflava* is only one isolate that produced catechols-type 4.12±0.90 μg/ml. In addition, 10 endophytic actinomycetes showed protease activity ranging from undetectable to 8.16±0.15unit/ml. Genetic relatedness amongst these isolates was determined base on Random amplified polymorphic DNA (RAPD). This methodology generated specific patterns corresponding to particular genotypes and the genetic diversity of isolates from each site was not significant. However, further investigations are needed to understand the other forms of relationships between endophytic actinomycetes and host plants that will be applied to agricultural fields.

**Keywords** Endophytic actinomycetes; IAA-3-acetic acid; Siderophores; Random amplified polymorphic DNA (RAPD)
Molecular properties and significance of phosphoenolpyruvate carboxykinase in a ruminal bacterium, *Streptococcus bovis*

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**Background**

*Streptococcus bovis* is usually a major lactate-producing bacterium in the rumen, and often proliferates when ruminants are fed diets containing large amounts of readily fermentable carbohydrates, such as starch. Thus, *S. bovis* sometimes causes rapid acidification in the rumen as a consequence of excessive lactate production, and is thought to contribute to the progress of rumen acidosis. Therefore, it is desirable to control the growth and metabolism of *S. bovis* so as to produce lactate at an adequate rate. Since *S. bovis* metabolizes sugar through the Embden-Meyerhof glycolytic pathway, the control of the glycolytic flux in *S. bovis* has attracted considerable attention in connection with the prevention of rumen acidosis. In this study, we focused on the significance of phosphoenolpyruvate (PEP) carboxykinase (PCK, EC 4.1.1.32) in *S. bovis*. This enzyme generally catalyzes the conversion of PEP to oxaloacetate (OAA) in bacteria, but also acts to generate PEP for gluconeogenesis by the reverse reaction in some bacteria. Therefore, we examined the molecular and enzyme properties of *S. bovis* PCK, and factors affecting PCK synthesis. In addition, the role of PCK was also examined by constructing a pck-disrupted mutant.

**Results**

We identified and characterized the PCK gene (*pck*) and flanking sequences from *S. bovis*. A BLAST search indicated that the deduced amino acid sequence of *S. bovis* PCK showed approximately 50% identity with those of other bacterial PCK proteins. Sequence analysis and mRNA analysis indicated that *pck* was transcribed in a monoeirstronic fashion. The level of pck-mRNA was higher when cells were grown on lactose than on glucose, suggesting that PCK synthesis is stimulated when the growth rate is low. The pck-mRNA level was higher in a mutant lacking cepA, which encodes the catalytic control protein A (CcpA), than in the parent strain, suggesting that pck transcription is suppressed by CcpA. Recombinant His-tagged *S. bovis* PCK was purified as a single protein, verified by SDS-PAGE. PCK activity was detected when the recombinant protein produced from *S. bovis* pck was added to the assay mixture, confirming that the pck encodes PCK. *S. bovis* PCK showed oxaloacetate (OAA)-decarboxylating activity, but no PEP-carboxylating activity (reverse reaction). The cell homogenate of *S. bovis* had high pyruvate carboxylase activity, but no PEP carboxylase activity was detected. Thus, OAA appears to be produced from PEP via pyruvate in *S. bovis*.

To examine the significance of PCK in *S. bovis* growth, the pck gene was disrupted by replacing the entire pck gene by an erythromycin resistance gene. Disruption of *pck* was confirmed by Southern-blot and PCR analysis. As expected, no PCK activity was detected in the pck-disrupted mutant (JB1-pck). In glucose-limited growth medium, the growth rate of JB1-pck was significantly lower than that of its parent, JB1, and the OD600 value at growth cessation for JB1-pck was also lower than that for JB1, showing that PCK affects growth. The ratios of formate to lactate produced by JB1-pck and JB1 in 1 h were not significantly different, suggesting that disruption of *pck* has no effect on the fermentation pattern during exponential growth. Thus, depression of growth does not seem to be caused by a change in the fermentation pattern. When JB1 was grown in a medium containing ammonia as the sole nitrogen source, the lag phase was prolonged and cell yield was decreased. This result suggests that it takes a while to initiate amino acid synthesis, and considerable energy is used for this purpose. Much more pronounced effects were observed when JB1-pck was grown, suggesting that PCK is involved in the initiation of growth including the induction of amino acid synthesis and energy metabolism.

**Acknowledgments**

This study was supported in part by a Grant-in-Aid for Scientific Research (No. 20780196) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (MEXT).

**Keywords** oxaloacetate production; phosphoenolpyruvate carboxykinase; rumen bacteria; ruminal acidosis; *Streptococcus bovis*

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Multiple associations involving ectomycorrhizal and endomycorrhizal fungi, nitrogen fixing bacteria and the leguminous species *Dimorphandra wilsonii*, a threatened species from the Brazilian Cerrado

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*D. wilsonii* is a Caesalpinioideae leguminous tree, native to the Brazilian Cerrado (Savannas type) and is currently threatened of extinction. Nowadays, there are only eleven of these trees that have survived in this region of Paraqueba and Lagoa Santa in the Minas Gerais state. Mutualisms associations between plant and soil microorganisms are strategies closely linked to the adaptive success of plant species. This study aimed to evaluate the presence of symbionts in the roots of *D. wilsonii*. Surprisingly there was a concomitant occurrence of nitrogen fixing bacteria (indeterminate nodules), ectomycorrhizal and endomycorrhizal fungi (AMF) found. The Bradyrhizobia was the dominant gender found in plant nodules. The dominant AMF families in rhizosphere were Glomaceae and Acarosporaceae which were found similarly distributed along of the roots. The root colonization assessment of arbucellular mycorrhizal fungi (AMF) was around 30% and there was a predominance of fungal structures related to the Acarosporaceae family. In contrast there was an abundant ectomycorrhizate colonization found in all the plants analyzed. Morphological analysis of AMF spores from rhizosphere soil confirmed the dominance of *acarospora* species. The multiple infection is a rare event, and may be related to the adaptive strategy for the survival of this species.

**Keywords**: Arbuscular mycorrhiza fungi, Nitrogen fixing bacteria, Ectomyorrhiza, Leguminous species
Mycobiota predominant and aflatoxins content in shell and shelled Brazil nuts

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Brazil nuts (Bertholletia excelsa Humb. and Bonpl.) are an important product of the Brazilian Amazon. Currently, its marketing is compromised by the high incidence of aflatoxins (AF). The most known naturally occurring AF are named AFB1, AFB2, AFG1, and AFG2. This study aimed to identify the potentially aflatoxigenic mycobiota associated with shelled Brazil nuts and with the shells, and to determine which one of these fractions contributes to aflatoxins (AF) contamination, since that official method use integral Brazil nuts samples to AF test. Samples of Brazil nuts were collected from the agro forestry system production area in Amazonian rain forest, in Brazil. These samples were split in shell and shelled nuts, and the total count of Aspergillus spp. was analysed after sanitation (sodium hypochlorite 1% / 10 minutes) and without sanitation, by plating AFPA medium, for 7 days, at 25 °C. The isolates identified as Aspergillus spp. were plated in YES medium (5days at 25°C) for determination of the aflatoxigenic potential by agar plug technique. To analyze AF, 500 g samples were milled and were extracted with chloroform. The chromatographic analysis was performed by HPLC-FD system in an isocratic mode (Waters pump W600, Waters module auto sampler W717, Fluoresce detector W2475 and column Waters X-Terra (4.6x150mm and 5μ – RP18)). The mobile phase was water mills-Qacetoniitride/methanol (60:0.150:150 v/v) and the injected volume was 5μL both to standards and samples. The average incidence of infection from Aspergillus spp. in samples Flavi, Nigri and Circumdati were 48%, 8% and 1%, respectively. The sanitation treatment reduced the fungi counts. There were AF production by fungi isolated from both types of samples, 30% of the samples were positive for AFB1, AFB2, AFG1, and AFG2. Concerning the Brazil nuts AF analysis, it was observed that the concentration of AFB1, and AFG2 obtained were higher than AFB2 and AFG1. The AFB1 content was 35.281 and 1.782 μg/Kg in shelled Brazil nuts and shells, respectively. AFB1 and AFG2 were detected only in shelled samples. The HPLC-FD presented limits of detection (LOD) and quantification (LOQ) of 0.2 and 0.4 μg/kg, respectively.

Keywords: mycotoxins, food safety, fungi, Bertholletia excelsa

N2O and N2 emissions from pasture soils differing in pH – does the linkage between the gas fluxes, denitrifying activity and size of the denitrifier community exist?

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Denitrification is of environmental concern because, together with nitrification, it is the main biological process responsible for N2O emissions. N2O is a potent greenhouse gas and after some reactions in the stratosphere it can also cause the destruction of stratospheric ozone. Both, the amount of denitrification end products (N2O and N2) evolved, and the N2O (N2+ N2O) ratio, are important in understanding, predicting and mitigating NOx fluxes from soils. Soil pH is one of the most important factors influencing both denitrification and N2O production. In general, denitrification rate increases with increasing pH values, while the N2O (N2+ N2O) ratio decreases. This relationship has already been well characterized in laboratory experiments, but not verified in the field because of methodological limitations for in situ measurement of N2 emissions. Soil pH is also an important factor influencing denitrifier community composition, which can be an important driver of denitrification activity and N2O emissions. The objective of the present study was to explore the effect of changes in soil pH on in situ N2O and N2 emissions and on denitrifying enzyme activity. In addition, we also investigated whether differences in the N-gas fluxes could be related to the size of the microbial community possessing different denitrification genes.

We established a field experiment situated in a grassland area in South Bohemia, Czech Republic, where we manipulated the soil pH. The field experiment consisted of three treatments which were repeatedly amended with KOH solution (alkaline soil), H2SO4 solution (acidic soil) or with water (pH-natural soil) over 10 months. At the site we determined field N2O and N2 emissions using 15N gas-flux method. Soil samples were collected for determination of denitrifying enzyme activity (DEA) and for determination of the size of the denitrifying community by quantitative PCR of the narG genes and also to the nirS, nosZ, and nirK denitrification genes. The total bacterial community was quantified using 16S rRNA as molecular marker.

Manipulation of soil pH via the application of acid or alkaline solutions resulted in a significant change in the soil reaction: pH 5.5, 6.8 and 7.7 for the acidic, pH-natural and alkaline soils, respectively. DEA and N fluxes in situ were highest in the alkaline soil and lowest in the acidic soil, but we did not find any differences in N2O production or emissions between the pH treatments. On the other hand, the N2O (N2+ N2O) ratio was the highest in the acidic soil and the lowest in the alkaline soil. The total N-fluxes in situ significantly correlated to DEA and the N2O (N2+ N2O) ratio in the field was significantly correlated to the N2O (N2+ N2O) ratio calculated from the DEA assay. For all denitrification genes and the 16S rRNA gene, the highest gene copy numbers were observed in the pH-natural soil. However, the abundance of none of the denitrification genes was correlated to total N-fluxes in situ and only the abundance of the nirS gene was correlated to DEA. The N2O (N2+ N2O) ratio was negatively correlated to the abundance of the nirK and narG genes and also to the nirS, narG, and nosZ genes. We found a positive correlation between the nirS and nosZ gene copy numbers and also to the nirK gene copy numbers and the N2O (N2+ N2O) ratio, which remains to be explored. However, in this study, the proportion of denitrifiers capable to reduce the N2O did not seem to have a role in determining the N2O (N2+ N2O) ratio. It is crucial in future studies to continue to bridge the gap between studies of denitrifier ecology and of N2O fluxes for a comprehensive understanding of the role of denitrification in determining not only total denitrification rates but also the nature of the denitrification end products. This work was supported by the research grants AV0Z60660521, MSM 6007668081, LC 06066 and IAA600660605, and by the Barrande Programme 2-07-26.

Keywords: denitrification; pH; soil; N2O; emissions; 15N; denitrifying enzyme activity; qPCR; narG, narA, napA, narL, nirK, nosZ.
Nematicidal activity of Solanum sisymbriifolium and S. nigrum extracts against the root-lesion nematode Pratylenchus goodeyi

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The root-lesion nematode Pratylenchus goodeyi, a parasite of banana plants, is very frequent in Madeira Island (Portugal) affecting culture development and consequently the production, with economical damages. To control phytoparasitic nematodes is common resorting to phytopharmaceutical products of high toxicity to animals and environment in general. In order to find solutions less aggressive to the environment and to man, different alternative pathways to chemical products are being studied. The nematicidal potential of some plants and its application have been analysed and different plant parts have been tested to identify the possible origin of the nematicidal substances. Recently it was demonstrated that the incorporation of Solanum sisymbriifolium and S. nigrum, in soil, improve banana plant growth by direct action through exudates release with nematicidal effect and by indirect action contributing to promote antagonist development and to turn rhizosphere not favourable to the nematode.

The aim of this work was to identify the organic components of S. sisymbriifolium and S. nigrum with nematicidal properties and to determine the effect of those components on P. goodeyi. In order to guarantee the total components extraction, an extraction sequence of at least 10 hours each from the dried plants was used. The chosen solvent sequence was: dichloromethane, acetone, ethanol and at last, water. The mortality of P. goodeyi in some extracts was significant being the water extracts the most effective. The water extracts from dried and fresh plants confirmed the mobility and mortality effects on P. goodeyi shown on the water extracts. According to the results both plants have in their composition chemical components that can be found in water, which affects the mobility and mortality of the root-lesion nematode. Therefore, S. sisymbriifolium and S. nigrum have potential to be used as a natural and environmentally friendly nematicide to control the root-lesion nematode, P. goodeyi. Further studies are being done in order to identify the active nematicidal components present in the water-soluble extract.

Keywords Banana plant; root-lesion nematode; Solanum; Nematicidal activity

Nitrifying microorganisms biodiversity in different soils types of the European part of Russia.

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Nitrifying and denitrifying organisms are important in removing fixed nitrogen pollutants from ecosystems, the NO and N2O produced by these processes are greenhouse gases. Central process in the global nitrogen cycle is the microbial ammonia oxidation, the first and rate-limiting step of nitrification. It was long time believed that microbial ammonia oxidation is solely performed by Bacteria and only bacteria possess the amoA gene encoding ammonia monooxygenase, the key enzyme of nitrification. Recently, this concept has been changed. The isolation of ammonia oxidizing archaea from marine aquatic ecosystems (Könneke et al., 2005) indicate that archaea may play an important role in nitrification. The aim of the present work was estimation nitrifying microorganisms biodiversity in different soils types of the European part of Russia. For this purpose it was elaborated the methods of molecular detection key functional genes ammonia-oxidizing microorganisms and the comparative analysis of results amplification of DNA isolated from soil samples of different ecosystems.

For our molecular investigations it has been chosen the novel gene encoding ammonia monooxygenase (amoA) in Bacteria and Crenarchaeota. The bacterial amoA gene was amplified by PCR with primers amoA-1F and amoA-2R (Rothhauwe et al., 1997) and the archaea amoA gene – with primers CrenAmoA1F and CrenAmoA1R (Könneke et al., 2005).

Soils were sampled from different bioclimatic zones of the European part of Russia. Four methods by straight lysy were used for each soil sample. All this methods DNA extractions were successful for amplifications 16S RNA genes. However, amplifications of extracted DNA were successful for amplifications amoA functional genes only with Power Soil DNA Kit (MO Bio).

By PCR amplification analysis it was found ammonia oxidizing bacteria in soddy-podolic soils and grey forest soils (under forest ecosystems); in chernozem and chestnut soil (under prary ecosystems).

For estimation diversity of ammonia-oxidizing bacteria received PCR products were divided by method of molecular cloning with the help of sequences and phylogenetic analysis. The comparative analysis of results from received clones libraries from soil samples and enriched cultures has shown that in soddy-podolic soil ammonia-oxidizing bacteria was represented by Nitrosospira while in enriched cultures - by Nitrosospira and Nitrosovibrio. In chestnut soil were identified representatives of Nitrosospira, Nitrosovibrio, and in enriched cultures – Nitrosospira, Nitrosovibrio.

It was developed the report of amplification which gave possibilities to receive a stable product of the fragment gene amoA of ammonia oxidizer archaea. The given report is updating before the published methods (Könneke et al., 2005, Dorador et al., 2008) with change temperatures, time of elongation and quantities of a DNA matrix. This products of amplification were isolated from soil horizons of soddy-podolic soil on 5-10 and 40-50 cm depths.

As a results of our work it was selected the primer systems and conditions of amplification for detection the fragment gene amoA in β-proteobacteria and Crenarchaeota. The comparative analysis has shown that the community structure of nitrifying microorganisms in soil samples and enriched cultures were divided. So that in the study of nitrifying microorganisms biodiversity it is necessary to use comparative analysis clones library gene amoA in soils and enriched culture.

References


Nodulation process and nitrogen fixation effectiveness in field beans (Vicia faba)

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The inoculation of the legume seed material with active nitrogen fixing bacteria strains before sowing has a significant role for the increase of the legume yield. Inoculation can improve crop yields in cases where appropriate rhizobia are not present in the soil or the soil contains a significant proportion of nonnodulating or ineffective nitrogen-fixing strains. The aim of the investigation was to detect the effectiveness of *Rhizobium leguminosarum* strains in field beans at different soil microbiological activity. The experiment was conducted at the Institute of Soil and Plant Sciences of the Faculty of Agriculture of the Latvia University of Agriculture. The field beans (*Vicia faba* L.) cultivars ‘Ada’, ‘Lielplatones’, *Rhizobium leguminosarum* bv. *vicia* strains N° 110; 408; 501 and 2 types of soils were used in vegetation pot experiment. Obtained results in cultivar ‘Ada’ and ‘Lielplatone’ showed that used *Rh. leguminosarum* strains resistance on streptomycin decrease in both soil types from the anthesis stage forward. For the cultivar ‘Lielplatone’ the highest resistance on streptomycin showed strain N° 110, but for cultivar ‘Ada’ strain N° 408. The shoot fresh mass, dry matter, pod number, weight, dry matter and accumulated nitrogen depended on used Rhizobium strain and soil features. The fingerprintings show significant difference between *Rh. leguminosarum* strains.

**Keywords** *Rhizobium leguminosarum*, field beans, nodulation, nitrogen fixation

Nucleotide Sequence analysis of the fusion protein gene of Newcastle disease viruses isolated from chicken in Iran

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In this study 9 newcastle virus (NDV), obtained from Iranian industrial chicken-farms, were isolated. A piece of 1349 nucleotide possessing virus’s F protein cleavage sits was sequentially determined. Amino-acids of F protein in isolated viruses were phylogenetically compared with previous viruses in Iran and the rest of the world. 96.5-100% of similarity was observed among isolated viruses in different provinces. The nucleotide sequence of the piece was analyzed considering encoding sequence of F protein in NDV-existing in gene bank by using blast software. The highest nucleotide similarity with strains of Italy/3286/00 and sterna/astr. Amino acids and F protein cleavage sits of these viruses is quite the same and RRQRRF. They did not show any change to the previously isolated viruses in Iran, but in 3 viruses out of 9. Amino acid position 265 changed from serin to glycin. Phylogenetic analysis indicated that all bear common source as previous viruses and they were included in a separate group along with Russian, Italy, Ira, Turkey, Saudi Arabia, Kuwait, Kazakhstan, Lebeman, South Korea, Japan, and South Africa viruses.

**Keyword**: newcastle virus, cleavage sits, Phylogenetic analysis, F protein
Occurrence of Methicillin-resistant Staphylococcus aureus at a Dairy Farm

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Methicillin resistant Staphylococcus aureus (MRSA) is a significant cause of human and veterinary infections. There were described cases of the MRSA transfer from humans into animals during veterinary treatments. For now, its occurrence in the dairy cows is fortunately sporadic.

There were 120 bulk milk samples examined, issued from different cow farms in Czech Republic. Single sample was identified as MRSA positive. Consequently, individual milk samples (mixed from the all four teats) were examined in the relative farm from 70 cows. In addition, samples (swabs from the rhino-pharynx, nose and the navel surroundings) from three dairymen were taken. Samples have been investigated for occurrence of mastitis pathogens, where identification of the MRSA was targeted. All together 32 bacterial species have been identified. The most frequent species found were as follows: Staphylococcus aureus (13; 41%), further Staphylococcus haemolyticus (3; 9%), Streptococcus uberis (3; 9%), Staphylococcus epidermidis (2; 6%), Staphylococcus xylosus (2; 6%), Streptococcus faecalis (2; 6%), Streptococcus porcinus (1; 3%), and Aerococcus viridans (1; 3%). Five isolates (10%) have been identified just genus-specifically as Streptococcus spp. In all S. aureus species oxacillin sensitivity (OX 5) has been found, determined by means of diffusion method. Five S. aureus have been found as oxacillin resistant, preliminarily signed as MRSA. At the all S. aureus strains identified, PCR-amplification has been performed towards the mecA gene detection. Positive identification has been in 3 S. aureus strains, which were oxacillin resistant, as well. These strains have been identified as MRSA. Results from human samples were negative.

This work has been granted by projects MZe NAZV QH81111, INGO LA 333 and MSM 267846201.

Pathogenic bacteria can produce exopolysaccharides and use them as carbon source under stress conditions: the case of Erwinia amylovora

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Bacterial exopolysaccharides (EPSs) have long been related in several bacterial species with cell protection under nutrient scarcity prevailing conditions in nature, and also against metals. However, the information about the protective role of EPSs in plant pathogenic bacteria under adverse conditions is still very scarce because EPSs have been mainly studied as virulence factors. The bacterium Erwinia amylovora, a highly virulent and necrogenic pathogen, causes the devastating fire blight disease in several pome fruits and ornamental rosaceous plants, being the responsible of great economic and commercial losses. Fire blight still remains as a serious threat for agriculture due to its difficult control, associated with the ability of E. amylovora to persist in nature.

In the plant environment, nutrient scarcity is imposed in leaves when Er. amylovora is in the epiphytic phase, or even in the endophytic phase when the host plant is in dormancy. Besides, copper is still widely employed to control fire blight, especially in the European Union, since the use of antibiotics is forbidden in plants. In spite of these two common adverse conditions in the plant environment, the role of E. amylovora EPSs to face these stresses had not been explored so far. Then, we have investigated whether amylovoran and levan, the major EPSs of E. amylovora, could be used as carbon sources under starvation conditions in the presence of copper, using EPS-deficient mutants (AMY- and LEV-) in comparison with wild type (wt) strains. Carbon-free AB mineral medium plus 0.005 mM Cu2+ ions was used to assay the simultaneous effect of carbon deprivation and copper, whereas AB with copper but supplemented with amylovoran or levan extract at 0.2% was used to evaluate the use of each EPS as carbon source under these stresses. In all cases, the culturability of E. amylovora was monitored on nutrient agar plates throughout six months at the same time that EPS levels were periodically quantified.

In AB with copper but without EPS extracts, the culturability of all E. amylovora assayed strains progressively decreased until drop below the detection limit (1 cfu/ml), although much more quickly for EPS-mutants than for wt strains. In contrast, when amylovoran or levan were present, the culturability was extended in all strains over the six-month period of study, at the same time that EPSs were depleted, although in greater extent in EPS mutants than in wt strains. Therefore, the proportion of non-culturable cells induced by starvation and copper stresses was significantly reduced by the use of supplemented EPS extracts as carbon source. This phenomenon has been reported in starved cells of some bacterial species, but not in plant pathogens, nor in the presence of copper. Further, culturable numbers were always significantly higher with levan extract than with amylovoran extract, indicating a preferential use of levan over amylovoran as carbon supply by the stressed E. amylovora cells.

Overall, the use of E. amylovora EPSs as carbon source under deprivation conditions, even in the presence of toxic copper ions, could explain, at least in part, the frequent persistent infections in copper-treated plants, and give new insights into the survival strategies developed by the pathogen to persist in nature.

Keywords: fire blight; copper; EPS; amylovoran; levan; levanase; starvation; culturability; carbon supply.
Phenotypic characterization and the application of the rep-PCR technique in the study of new strains of *Bacillus thuringiensis* in the South of Brazil

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Worldwide, the *Bacillus thuringiensis* (Bt) is now the most widely used bio-pesticide. Its toxicity derives from the production of specific proteins (delta endotoxins) during sporulation. In this study, 26 Bt strains from South Brazil were analyzed by phenotype and molecular testing of sequential amplifications of repeated sequences (REP-PCR) to evaluate the intra-specific similarities between the strains, and to determine the internal homogeneity.

The 26 Bt strains were isolated in the rice-growing regions in the South of Brazil (Fronteira Oeste-FO, Campanha-C, Litoral Norte-LN, Litoral Sul-LS and the Depression Central-DC) and were supplied by the UNISINOS BRB. For the phenotypical tests with organic composts, the strains were grown in a simple glucose growing environment (MUG) for 48 hours at 33°C, subjected to growth in 100% ethanol (150 and 250μL), rotenone 4 % (40 and 60μL), phenol 14% (40 and 60μL) and xylen 40% (150 and 250μL), adding the corresponding quantity of each compost in 10μL of MUG. The growth was determined after five days in B.O.D at 30°C. In the tests with chemical pesticides-Epipol (4μL), Pirazosulfluronetil (4μL), Quinclorac (0.02 g), Propanil (255μL) and Akoazitoxina (26μL), the lineages were grown in Petri dishes with MUG for 48 hours at 33°C and the recommended amounts of each pesticide were deposited at points 5 cm apart, in B.O.D at 33°C. After incubation for 24 hours, the distance between points where the growth had been inhibited. For the analysis of the protein profile, in SDS-PAGE (12%), the strains were cultivated until cellular lise, the bacterial suspensions were centrifuged (at 10,000rpm; 10°C; 20 min) and the pellet was diluted in 1ml de sterilized water - 75μl of this was added to 25μl of buffer and heated to 100°C for 10min. The solubilized proteins of each strain were centrifuged (10,000 rpm; 5min) and the supernatent was applied to the SDS-PAGE (12%) of strains containing isolates with degrees of similarity ranging from 60% to 85%. The results of the tests with the new strains of *B. thuringiensis thuringiensis* 4412 strain and the DNA negative-free control. The amplification was realized under the following conditions: denaturation (4 min at 94°C; 35 cycles at 94°C for 30sec), ringing (30sec at 45°C), extensions (1min at 72°C) and 10 min of extensions at 72°C. The amplification products were analysed in an agarose gel at 10% with a 1-kb DNA ladder (Invitrogen). For the statistical analysis a similarity matrix between the lineages was estimated using the software NTSYS package and the dendograms were constructed by the UPAGMA non-weighted pair group method.

The Bt strains analysed via the phenotypical tests generated similar phenograms for the chemical pesticides, organic composts and SDS-PAGE, presenting distinct protein classes. In these analyses, five different groups (G) were formed in each geographic region (G1- DC; MW: 50, 65 and 130 kDa; G2- C: MW: 25, 40, 55 and 100 kDa; G3 - LN; MW: 40, 50, 80 and 110 kDa; G4 - FO: MW: 25, 50, 80 and 130 kDa; G5 - LS; MW of 25, 50, 65, 80 and 130 kDa) of strains containing isolates with degrees of similarity ranging from 60% to 85%. The results of the tests with REP-PCR demonstrated polymorphism between the strains, that is, the number of bands found for the group of primers of all the strains varied between 3 and 9 for each sampled region and the bands sizes fell between 0.4 and 3.4 kb. The topology of the dendograma generated was similar to that generated by the other tests, making it possible in this way to separate the isolates in accordance with the geographical region.

The present study demonstrates the applicability of phenotyping processes allied with REP-PCR, and shows them to be highly selective, rapid and capable of identifying distinct Bt lineages. The tests demonstrated many similarities with the new strains of Bt obtained in the South of Brazil indicating a high degree of clonability between the rice culture regions, probably associated with asexual reproduction and/or ecological speciation. These characteristics are important for the application of these strains in a system of integrated management of rice cultivation.

Keywords: *Bacillus thuringiensis*; rep-PCR; SDS-PAGE; Southern Regions of Brazil.

Phylogenetic characterization of *Beauveria bassiana* isolates originated from different insect hosts

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*Beauveria bassiana* is an entomopathogenic fungus that exists naturally in soil and used in biological control of various agricultural pests often attack by insects with a wide range of hosts. The systematic and phylogeny may have practical effects in entomology. Phylogeny is the study of evolutionary relationships of organisms and systematically uses the results of phylogeny to construct systems of classification. Today, the big question in relation to the concept of morphological species in *Beauveria* is the pattern of variation morphophysiologically is related to the phylogeny and the morphological species are cryptically diverse, that is, if these species are reproducese isolated from one another, but morphology is very similar. The study aimed to assess phylogenetically through the ribosomal Internal Transcribed Space (ITS) of *Beauveria*. These five isolates of fungi were obtained from the collection of the laboratory of biological control of the Instituto Agronômico de Pernambuco-IPA where each isolate received a number. Each was isolate from different host and different geographic origin as described below: IPA145 (Coleoptera: Curculionidae; IN = indeterminate), IPA148 (Coleoptera: Coccinellidae, IN), IPA223 (Lepidoptera: Pyrillidae, Paraiba-BR), IPA225 (Orthoptera: Acrididae; Recife / PE-BR), IPA226 (Coleoptera: Curculionidae; Cabo de Santo Agostinho / PE-BR). For DNA extraction the mycelium was ground 25 ml of liquid BD medium for 72 hours from 28°C without agitation and macerated liquid nitrogen and used the DNAeasy Blood & Tissue Kit (QIAGEN) according to the manufacturer. The ITS region was amplified using the primers ITS1 and ITS4. The sequencing was performed on the platform of DNA sequencing of CENARGEN-EMBRAPA. The sequences were aligned in the program BioEdit v. 7.0.0 and performed phylogenetic analysis with the program Mega version 4.1. It is also used sequences from the database of genes (GenBank) to compare our sequences (AY532046 and 532,044 isolated from *B. bassiana*). The results showed that all the isolates except IPA145 presented as rare and different monophyletic isolates found in NCBI. Whether they are isolated from hosts of the same order as Coleoptera, Lepidoptera, Orthoptera, no influence on the genetic similarity of less than 60%. We can conclude that the results on the molecular phylogeny of *Beauveria* is still very confusing due to the small current taxonomic understanding of this genera. He also noted that the phylogenetic diversity can indicate a history of cryptic diversification and molecular analysis are useful tools for the assessment of species, and elucidating the evolutionary and ecological history of these genera.

Keywords: ITS, *Cordyces bassiana*, evolutionary relationships, cryptic species, phylogenetic diversity.
Polybiotrophy of *Serratia marcescens*, a causative agent of an onion disease in arid zone of the South of Ukraine

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*Serratia marcescens IMBG291* was isolated from internal swollen leaf bases of naturally infected onion bulbs. Inoculation of slices from symptomless onion bulbs with the bacterial isolate resulted in tissue decay. However, the isolate DNA did not recognize specific primers designed for plant pathogenic strains (Zhang et al., 2005). Inoculation of *Arabidopsis thaliana* (L.) Heynh, onion (*Allium cepa* L.) and lettuce (*Lactuca sativa*) seeds resulted in biomass promotion of symptomless plants. The isolated bacterium exhibited a low potency to induce systemic resistance in *Arabidopsis* plants against *Pseudomonas syringae pv. tomato* 3008. The strain IMBG291 produced red pigment under specific conditions, in contrast to pathogenic strains, and the pigment production was regulated by environmental factors. A mobile genetic element, the integron, has been described in genome of phytopathogenic *S. marcescens* for the first time. The gene cassettes harbored by the integron have been represented with the promoterless gene encoded formiminoglutamate deiminase, hydrolase that takes part in glutamate metabolism, the gene coded for ascorbate-specific phosphotransferase system enzyme IIC, inner membrane protein, and with additional three senseless noncoding sequences flanked by 59-bp element. The integron may provide additional possibilities for utilization of a wider range of energy sources and better accommodation to changed environment for polybiotrophic *IMBG291* strain.

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Polyphenol oxidase in golden chanterelle (*Cantharellus cibarius*) mushroom

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Edible mushrooms have been traditionally recognized for their nutritional and medicinal value, and countless modern investigations have reported highly nutritious content as well as therapeutic properties such as anticancer, hypolipidemic, anti-inflammatory and many others, in these organisms. Bioremediation benefits are also associated with mushrooms, going from degradation of various environmental pollutants to antiherbivores and insecticidal properties. The golden chanterelle (*Cantharellus cibarius*) is an edible ectomycorrhizal mushroom much appreciated for its flavor and quite beneficial for the health through its antioxidant, immunomodulatory, anti-inflammatory and antimicrobial properties. Because of its complex association with trees, sustainable production of the mushroom is tightly linked to forests preservation. *C. cibarius* is also remarkably resistant to insects and parasites and exhibits insecticidal properties. Edible mushrooms are characterized by a short shelf life due to post-harvest changes resulting from the activity of enzymes such as polyphenol oxidase (PPO) that is responsible for browning reactions during storage. PPO is also involved in wound healing and defense mechanisms in plants, including defense against herbivores. It is a bifunctional enzyme, widely distributed in prokaryotes, eukaryotes and plants, catalyzing, in the presence of oxygen, the o-hydroxylation of monophenols (cresolase activity) and subsequent oxidation of o-diphenol to quinone (catecholase activity). Although the active site of PPO is conserved, the amino acid sequence and characteristics of the enzyme such as substrate specificity and sensitivity to inhibitors, varies considerably among species. Because of its role in post-harvest quality loss and its various physiological functions, the purpose of this research was to study PPO activity in the golden chanterelle mushroom hitherto unreported.

The golden chanterelle (*C. cibarius*) was homogenized in phosphate buffer 0.01 M, pH 7 (containing 0.02% phenylmethylsulfonyl fluoride as protease inhibitor) and centrifuged successively at 3,000 g for 10 min and at 35,000 g for 30 min; the supernatant was homogenized and centrifuged successively at 3,000 g for 10 min and at 35,000 g for 30 min; the supernatant was used for the study. The observed limiting reaction rate was at least 60 times higher for p-cresol than for the other substrates that were oxidized. The enzyme exhibited differential activation by SDS and was sensitive to kojic acid, a potent PPO inhibitor, although the concentration required for 50% inhibition differed by up to 20 times, depending on the substrate used. Substrate preference, differential activation by SDS and differential sensitivity to inhibitors have been reported by others for PPO from various sources, illustrating the wide variability of the enzyme across species.

**Keywords** chanterelle; polyphenol oxidase; cresolase activity; catecholase activity; kojic acid; SDS activation
Population diversity of *Cryphonectria parasitica* in Croatia

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Ascomycete fungus *Cryphonectria parasitica*, one of the worst pathogens of sweet chestnut (*Castanea sativa*) has been destroying chestnut trees in Europe for decades. Asian chestnut species are well adapted to the fungus, but European chestnut succumbs to the infection easily. Mycelia enter tree bark through wounds and spread toward cambium, obstructing normal flow of nutrients and water through stem. As a result, the tree more or less rapidly dies. All European chestnut formations have been infected and the tanker problem has become a festering wound in European forests. However, few years after the accidental introduction of *C. parasitica* in Europe, a phenomenon called hypovirulence was observed – some of the trees infected were slowly healing from the disease, and the other were showing much weaker symptoms of infection. It has been determined that for this hypovirulence phenomenon a small double-stranded RNA was responsible. Soon, it was revealed that this dsRNA is a virus which was named *Cryphonectria* hypovirus (CHV1). Hypovirus can be transmitted from one fungus to the other with the consequence of converting formerly virulent strain of fungus to the hypovirulent one. This conversion occurs between compatible strains of fungus.

*C. parasitica* was introduced in Croatia in 1955, but only recently vegetative compatibility (vc) types of the fungus were determined. This is very important for genetic diversity studies and estimation of the incidence of fungal sexual reproduction which results in recombination and, as a consequence, in new vegetative compatibility types. A total of 18 different vegetative compatibility types were observed in Croatia, more than in other Balkan countries, but less than in Italy and Switzerland. Some of the vc-types observed (EU-1 and EU-2) are dominant in west, while EU-12 is dominant in the south of Europe. EU-12 was also observed only in eastern continental populations in Croatia, while EU-1 and EU-2 are dominant in western and coastal populations. Therefore, in respect to the occurrence of the main vc types, the *C. parasitica* populations in Croatia combine features of both north-western and the south-eastern European populations. Perithecia and both mating types of *C. parasitica* in approximately 1:1 ratio were found in all populations suggesting that sexual reproduction of the fungus is common in Croatia.

Microsatellite loci of *C. parasitica* were also analyzed in population studies. Preliminary results on eight microsatellite loci revealed the existence of as much as 37 different haplotypes in Croatia, also much more than in other Balkan countries (Bulgaria, Greece, Macedonia and Romania). Diversity indexes were similar to those determined by vc-typing of fungus. G2 values estimates showed strong genetic differentiation between coastal and eastern continental populations, and some Croatian mid-continental populations showed mixture of coastal and eastern microsatellite characteristics. When ANOSIM was performed, eastern populations significantly differentiated from the coastal, but mid-Croatian populations showed no significant difference from either coastal or eastern continental populations, also a strong indication of mixing of *C. parasitica* populations from north-west and south-east in Croatia. Multilocus linkage disequilibrium index also supports the hypothesis that *C. parasitica* populations in Croatia predominantly reproduce sexually.

These data show a potential threat of formation of new vc-types, thus making transmission of hypovirulence harder and natural biological control of disease more difficult then assumed. This problem deserves much attention especially in the coastal region of the country where the incidence of hypovirulence is low and human-mediated biocontrol is needed.

**Keywords:** biological control, genetic diversity, population studies

Potential for biocontrol of *Anthonomus grandis* using a chitinolytic extract of endophytic *Streptomyces* sp.

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The demand for cotton is consistent and steady making the production of cotton potentially one of the most financially stable commodities worldwide. About US$ 3–5 billion are spent on pesticides per year, and of that US$ 645 million are used in cotton production. Cotton boll weevil, *Anthonomus grandis* Boheman (Coleoptera: Curculionidae), is an important pest of cotton production in the Americas. This pest is controlled by chemical agents, nonetheless, chemicals are expensive and due to their broad-spectrum activity may disrupt predator and parasitoid populations as well as pests. Thus, biological and other control methods are encouraged in integrated pest management strategies, which require more selective pesticides to decrease the damage on cotton crops by boll weevil pest. The chitin, an insoluble linear polymer, is one of the major structural components of exoskeletons and cylindrical peritrophic membrane in the midgut of most insects. Thus, the degradation of insect chitin has long been considered an attractive target for insect control. The present study deals with the partial characterization of the chitinolytic extract produced by an known chitinolytic endophytic actinomycete (*Streptomyces* sp.) and the evaluation of this extract against *A. grandis*, the cotton boll weevil. Experiments were carried out using the high chitinase producer Streptomyces sp. A8 strain, which was previously obtained from *Citrus reticulata*. A chitinolytic extract produced by endophytic *Streptomyces* sp. (A8 strain), was biochemically characterized and then tested against *A. grandis*. The chitinase crude extract only from 5 days old cultures of A8 strain cultured in liquid minimum media supplement with chitin was partially characterized employing standard methods. The chitinolytic extract had the optimal temperature (maximum activity at 60°C) and pH from 4 to 9 (around 80% of relative activity) as well temperature and pH stability and inhibitors characterized. The filtered chitinolytic extract was added to an artificial diet for boll weevil (Figure 1). The boll weevil development from egg until adult stage was elongated and the statistical analysis showed strong genetic differentiation between coastal and eastern populations. Multilocus linkage disequilibrium index also supports the hypothesis that *C. parasitica* populations in Croatia predominantly reproduce sexually.

*Chitinolytic effects on development of boll weevil.* The percent of adult boll weevil was obtained from percent of eggs placed in the diet medium. The chitinolytic activity was the rearing activity compared. The statistical difference between the two curves and the regression equation was obtained by ANOVA using four replicate.

**Keywords:** chitinase; endophytes; *Streptomyces*; biocontrol; *Anthonomus grandis*

![Figure 1. Chitinolytic effects on development of boll weevil.](image1.png)

![Figure 2. Mortality of boll weevil under chitinolytic artificial diet.](image2.png)
Prevalence and Pathogenicity of Airborne *Fusarium* species in south east coast of Spain

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The prevalence of airborne *Fusarium* species was determined in the South East coast of Spain. Air-dust (downfall dust) was collected during September 2007, July, August and October 2008. Five different *Fusarium* species were isolated from the dust: *Fusarium oxysporum*, *F. solani*, *F. equiseti*, *F. dimerum*, and *F. proliferatum*.

Pathogenicity assays were conducted with 37 of the collected isolates of *Fusarium* species. Twenty isolates from the collected downfall dust: seven isolates of *F. equiseti*, five of *F. oxysporum*, four of *F. solani*, two of *F. proliferatum*, and two isolates of *F. dimerum*. And seventeen isolates from the dust carried with rain water (after evaporation): eight isolates of *F. solani*, four of *F. oxysporum*, three of *F. equiseti*, and two isolates of *F. proliferatum*. Results show differences in the pathogenicity of the isolates tested. Little pathogenicity was observed on tomato caused by *F. oxysporum*, *F. proliferatum* and *F. equiseti*, but none of the isolates of *F. solani* and *F. dimerum* were pathogenic on tomato. On *Cucumis melo L.*, two isolates of *F. solani*, and one isolate of *F. proliferatum* and *F. equiseti* caused significant decrease in seedling emergence. Pathogenicity of *F. solani* was also observed on *Sorghum vulgare* Pers.. One isolate of *F. proliferatum* produced an extensive pre-emergence damping off on cucumber (*Cucumis sativus* L.). On *Pisum sativum* L., all the *Fusarium* species tested produced an extensive pre-emergence damping off.

Almost all plants showed root rot when they were inoculated with different species of *Fusarium*, although fresh weights did not bring any information about the pathogenicity.

The evidence of long distance aerial dispersal of pathogenic strains of *Fusarium* species has to be taken into account as survival strategy for plant pathogens within the population dynamics as well as for plant protection strategies. *Fusarium* spores dispersed by wind from infected crops to new cultivated areas may overcome effective resistance.

**Keywords**: airborne mycoflora; aeromycobiota; biogeography.

Production of beer using sorghum and sorghum malt

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The production of beer using sorghum and sorghum malt was conducted.

Beer is an alcoholic beverage obtained from the fermentation of sugars, especially from barley, and barley-malt. Currently, starch from rice and corn is added to increase fermentable sugars in worts. Since many years ago, small and big breweries have been experimented using alternative grains instead barley, and among that we can find wheat, corn and rice. However the use of sorghum (Sorghum vulgare) for beer elaboration in an industrial plant was launched three years ago in USA. In this study, sorghum and sorghum malt were used as ingredients for beer production. The sorghum was milled with a roller mill and the malt was milled with a hammer mill. The wort was subject to mashing process and homogenized 10 g per l, then filtered after the fermentation. The sensorial properties like flavor, aroma, color, and body were evaluated. This study shows that sorghum could be used to beer production because it is cheap, safe and it is available during all year in our country.

**Keywords**: Sorghum, beer, fermentation
Production of Prodigiosin in Serratia marcescens PTCC1111 in Different Mediaes

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Abstract
Aim: Prodigiosins are bacterial secondary metabolites and are family of poly pyrrolyl red color pigments that are considered as antifungal and antiphytopathogenic factors. Prodigiosins are reported to have antitumor and anticancer properties. Due to the importance of the medical properties of this pigment, attention has been focused to increase its production. Therefore the present study aims to use various conditions to improve production of this pigment from the host bacterium. Its application are in pharmacology and in biological control of some plant pathogens.

Methods: In this study, Production of prodigiosin was achieved in LB and NB media, peanut and soybean powder and malt extract by standard bacterial strain Serratia marcescens PTCC111. To extract the pigment and estimate the level of production, the soluble was acidified by 4% 1 M Hcl in ethanol and then was centrifuged twice at 7000 r.p.m. for 10 min. The supernatant was used for prodigiosin production measurement.

Results: Results from experiments indicate that mentioned pigment produced in peanut medium and pH of 8 show highest prodigiosin production and malt extract medium with pH of 8 had the lowest pigment production.

Key words: prodigiosin, secondary metabolite, pigment, Serratia marcescens

Production of Prodigiosin in Serratia marcescens PTCC1111 in Different Mediaes and Study of Its Antimicrobial Effect as Biocontrol Some of Phytopathogenic bacteria

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Background: Prodigiosin are belonging of bacterial secondary metabolites and the family from polypirol red pigments. These pigments considerate as one of the antifungal and antipathogenic plants.

Material & Methods: Standard strain in this research is Serratia marcescens PTCC111 and phytopathogenic bacteria are including Agrobacterium tumefaciens Erwinia carotovora, E. amylovora, Xanthomonas campestris pv. campestris, pv. malvacearum, X. citri, Ralstonia solanacearum, Pseudomonas syringae pv. syringae and Ps. putida.

Results: Production of prodigiosin accomplished by mediums of NA, mutated LB and peanut. mentioned pigment extracted by 4% 1 M HCL in ethanol and its effect observed on above bacteria by antibiogram test with disk and cup plate method and measured the rate of growth inhibition zones.

Results: most effect was against Xanthomonas campestris pv. campestris with growth inhibition zone 33.5 and least effect was against Erwinia carotovora with growth inhibition zone 8.75.

Conclusion: the rate of prodigiosin production increase under influence of effective agents and comprise better effects that these effects is different by resistance and sensitivity of various bacteria.

Keywords: prodigiosin, Serratia, Agrobacterium, Erwinia, Xanthomonas, Pseudomonas
**Pseudomonas fluorescens** S1Pf1Rif increases plant tolerance to chrysanthemum yellows phytoplasma infection (“Candidatus Phytoplasma asteris”)

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Phytoplasma are prokaryotes belonging to the *Mollicutes* class. Knowledge of their biology is limited because they are non-culturable obligate parasites. Phytoplasmas are persistently transmitted by phloem-feeding insects, mainly leafhoppers of the family Cicadellidae, but the specificity of transmission, i.e. the relationships between leafhoppers and the pathogens they transmit, is still poorly known. Although Phytoplasma associated diseases are spread worldwide, strategies to limit their diffusion lead only to partial success. For some crops the losses are relevant and chemical treatments against insect vector are required by law, with a negative impact on environment and human health. In this context, the use of plant beneficial bacteria to control phytoplasmas has never been explored.

Bacteria living in the rhizosphere may influence plant growth and health by a number of mechanisms. Direct stimulation of plant growth are usually related to hormone (i.e. indole acetic acid, IAA) synthesis, mineral nutrition improvement (i.e. phosphate solubilization, nitrogen fixation) and modifications of root architecture. Indirect stimulation relies mainly on plant health improvement through the biocontrol of phytopathogens mediated by antibiotics, lytic enzymes and siderophores, the enhancement of plant tolerance to environmental stress by the production of the enzyme-1-aminocyclop propane-1-carboxylate (ACC) deaminase, lowering stress ethylene levels in plants, or the triggering of the induced systemic resistance (ISR). Hence, the presence of beneficial rhizosphere microorganisms, able to increase plant tolerance to biotic stresses, or to behave as biocontrol agents may represent a valid alternative for controlling phytoplasma diseases.

In the present work a model patho-system represented by *Chrysanthemum carinatum* (Schousboe) / chrysanthemum yellows (CYP) phytoplasma / its leafhopper vector *Macrosteles quadripunctulatus* (Kirschbaum) was used. CY phytoplasma is a strain of the “Candidatus Phytoplasma asteris” phytoplasma (16Sr-IB) which infects a number of dicotyledonous plants and is transmitted with different efficiencies by several species of leafhoppers.

The aim of this work was to analyze the effects of selected rhizobiotic bacterium, with characterized physiological traits, *Pseudomonas fluorescens* S1Pf1Rif, on daisies infected by CYP. Plant health was evaluated by measuring symptom severity, plant development and root architecture and discussed in relation to the quantitative determination of the titre and viability of CYP and to the qualitative description of phytoplasma cell morphology by transmission electron microscopy. CYP titre was measured by quantitative PCR (Q-PCR), and a new method for the evaluation of CYP viability in planta through Q-RT-PCR was developed. Reduced plant growth and root development were observed in CYP-infected plants. *P. fluorescens* S1Pf1Rif rescued plant growth reduction, consistently with the less severe and delayed symptoms observed. The phytoplasma titre in young apical leaves of CYP-infected plants, inoculated or not with the fluorescent pseudomonad, did not differ. However, CYP titre increased during time only in plants not inoculated with the strain S1Pf1Rif. Phytoplasma viability decreased over time, irrespectively of the presence of *S1Pf1Rif*. In fully developed leaves of CYP-infected plants inoculated with S1Pf1Rif, phytoplasma cells appeared often degenerated. Overall, the results indicate that *P. fluorescens* S1Pf1Rif was able to alleviate and delay the disease. The possible mechanisms involved in this symptom relief are discussed in relation to the physiological activities of the fluorescent pseudomonad.

**Keywords** copper; zinc; soil; poplar; cultivable bacteria; DGGE

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**Risk characterization of selected contaminants in sewage sludge: microorganisms, total phenols and heavy metals**

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Sewage sludge’s used in this study was obtained from residues resulting from the urban treatment of wastewater. The sewage sludge’s contain nutrients and organic matter that can provide soil benefits, and are widely used as soil amendments. They also have contaminants including heavy metals, pathogens microorganisms, total phenols and inorganic (metals) pollutants. The aim has been to generate ecotoxicological data and risk characterizations for selected microorganisms and hazardous substances in sewage sludge. The microbiological characterization indicated the presence of total coliforms and absence of fecal coliforms. The investigations with filamentous fungi isolated *Penicillium sp.*, *Chrysosporium sp.*, *Scedosporium sp.* and all of them are considering an opportunistic class C2, and no offer risk for health. The analysis of the phenol degradation indicated phenoloxidase production except, *Aspergillus sp.*. The results showed the ability of the microorganisms to degradate phenolic compounds. The chemical characterization of sewage sludge indicated: total carbon content 14.7833 mg/100 mg, and total phenols the value of 0.198 mg/l, respectively. The results indicated the presence of Ca, Mg, Al, Zn, Cu, Ni, Cd, Fe, Cr, and Mn, and the levels are according to the Brazilian law, except to Mn. The toxicity test carried out of the dried sewage sludge using *Artemia salina* higher toxicity was observed. The results suggested that the excessive fertilization and prevent unacceptable environmental effects or accumulation of contaminants, the use of sewage sludge must be regulated and controlled.

**Keywords** Sewage sludges; Microorganisms, Heavy metals; Total phenols; Toxicity
Role of *Pseudomonas fluorescens* containing ACC-deaminase and organic fertilizer on growth promotion of maize and sorghum under water stress field conditions

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Certain plant growth promoting rhizobacteria (PGPR) are known to mitigate the negative effect of biotic and abiotic stresses on growth and yield of plant crops through their ACC-deaminase activity. Field studies were conducted to evaluate the effects of *Pseudomonas fluorescens* containing ACC-deaminase and rock phosphate enriched organic fertilizer (OF) on growth of maize and sorghum under water deficit stress conditions. Water stress was created by skipping the irrigations at critical stages of plant growth. In case of sorghum trial 2, 3 & 5 irrigations while in maize trial, 3 & 5 irrigations were applied, considering 5 irrigations as optimum for plant growth. The results revealed that in case of un inoculated control (chemical fertilizer only), the growth of both crops significantly decreased as number of irrigations reduced from 5 to 2. However, inoculation with PGPR, *Pseudomonas fluorescens* substantially diluted the negative impact of less than optimum number of irrigations on growth of both the crops. Combined use of inoculation plus OF proved the most effective in promoting the growth of maize and sorghum under all the irrigations but impact was more obvious in case where irrigations were skipped i.e. in case of 2 or 3 irrigations. Interestingly, the growth parameters of both the crops were greater in response to inoculation under 2 or 3 irrigations than that recorded in case of uninoculated (chemical fertilizer only) treatments under maximum (5) irrigations.

Keywords: PGPR; organic fertilizer; water stress; cereals

Sequencing of the region of ribosomal internal transcribed spacer (ITS) of *Metarhizium anisopliae* in Pernambuco State

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*Metarhizium anisopliae* is a biocontrol agent that is capable of infecting a wide variety of hosts. Belonging to a genera of entomopathogenic fungi characterized as Deuteromycota: Hyphomycetes includes several fungi asexual. Currently there are three genera *Metarhizium* and nine varieties: *M. anisopliae* among which varieties are: *anisopliae*, *majus*, *lepidiotum* (*lepidiota*), *acradium*. The second is *M. flaviviride* which varieties are: *flaviviride*, *minus* *nauzae* (*lindosius*) and the third is called *M. album*. Some pathogens of insects have no restrictions on their hosts, infecting only a few species of insects, while others infect a wider range of hosts, if any of *Metarhizium anisopliae*. In 2001, *M. anisopliae* var. *majus* was identified as the anamorphic form of the fungus *Cordyceps brittebankisoides* through the ITS sequence (Internal Transcribed Spacer) and from these data considered one Ascomycota. This study aimed to elucidate the phylogeny of the isolates from the laboratory of biological control of the Instituto Agronômico de Pernambuco (IPA) - Brazil. All isolates except IPA217 were the same host order Homoptera: Cercopedeae (sugar cane spittlebug) and the host IPA217 grasshopper (Orthoptera: Acrididae). For DNA extraction the mycelium was ground 25 ml of liquid BD medium for 72 hours from 28°C without agitation and macerated liquid nitrogen and used the DNAeasy Blood & Tissue Kit (QIAGEN) according to the manufacturer. The ITS region was amplified using the *primers* ITS1 and ITS4. The sequencing was performed on the platform of DNA sequencing of CENARGEN-EMBRAPA. The sequences were aligned in the program BioEdit v. 7.0.0 and performed phylogenetic analysis with the program Mega version 4.1. It is also used sequences of the database of genes (Genbank) to assist in the classification by phylogenetic sequence of nucleotides of the ITS (EF489294, AY646386), *M. anisopliae* var. *lepidiota* or *lepidiotum*, FJ787313, *M. anisopliae* var. *acradium*, EU307926 and EU307906, *M. anisopliae* var. *anisopliae*; *M. flaviviride* AY375449, AJ099333 *Cordyceps brittebankisoides*; *M. album* AJ375446, AY847486, *M. anisopliae* var. *majus*. The results showed that the origin of the host was not of great importance for the phylogeny as the fungus IPA217 proved to be monophyletic branch as well as others that were isolates from the spittlebug cane sugar. The genetic similarity of fungi IPA213, IPA215 and IPA217 was higher than 92% with *Metarhizium anisopliae* var. *lepidiota*, *lepidota* who between them had a genetic identity of 100% showing that the different nomenclature is no sense. The same occurs with *M. anisopliae* var. *anisopliae* and a branch also monophyletic with 100% identity to access *M. anisopliae* var. *acradium*. However, this similarity than the other varieties are finding their earliest ancestors. The fungus IPA217 showed an identity of 94% with *M. flaviviride* and a similarity above 92% but also an identity of 94% with *Cordyceps brittebankisoides* and *M. album*. And the isolated IPA216 did most distanced himself from the other isolates showed to have been derived from an ancestor much older and an identity with *Metarhizium* of 61% and the isolate IPA217 can be an anamorph of *Cordyceps brittebankisoides* or *Metarhizium album*. We conclude that molecular analysis of the ITS region, the isolates are *Metarhizium* but with a different ancestry and that these fungi may be new varieties have not been identified for this it is necessary to further genetic studies, morphological and molecular characters of these isolates

Keywords: *Cordyceps brittebankisoides*, genetic identity, sugar cane spittlebug, phylogeny, ITS1, ITS4.
Silicate weathering potential of bacteria isolated from different soil profiles

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Microbial diversity contributes to soil functioning and can be used as biological indicator of soils healthiness and fertility. Heterotrophic bacteria play a major role in the availability of nutrients in soils. They participated directly or indirectly to weathering of primary materials (mainly silicates). These bacterial processes lead to leaching and release of essential elements, which (1) sustain plant growth, (2) determine the chemistry of soil solutions and exchange complex (3) participate to the formation of secondary minerals. In aerobic conditions, the two bacterial major processes involved in silicate weathering are acidolysis (i.e. proton promoted dissolution) and complexolysis (i.e. ligand promoted dissolution). However, these processes are generally studied and quantified without taking into consideration the functional bacterial diversity implicated in silicate weathering in soil and the environmental factors (such as parent rock materials, the climate, the vegetation...).

At a profile scale, soils represent mainly “oligotrophic” and micro-structured environments (microhabitats) where physicochemical conditions, organic matter and mineral contents can change rapidly over time. Bacteria must adapt to these changes and develop strategies to their survival. In contrast, the rhizosphere of plants provides microhabitats rich in carbon substrates (exudates of plants). Few studies have investigated on silicate weathering potential of bacterial strains isolated from rhizosphere or mycorrhizosphere (1,2,3). They concluded that these bacteria have high weathering ability in contrast to those isolated from bulk soil, and that efficient silicate weathering bacteria were able to adapt to nutrient-poor conditions. However, these works were only focused on the first ten centimeters of soils.

The aim of this study was to determine the weathering potential of bacteria isolated from different soils at different depths to establish relationships between key habitat determinants and bacterial weathering strategies (i.e. acidolysis and complexolysis processes).

Miniatursised bioassays using phlogopite as silicate mineral target were performed in aerobic conditions in order to determine the weathering ability of cultivable bacteria extracted from each horizon of contrasted soils in northeastern France (i.e. gleysic luvisol, calcareous luvisol, colluvial calcareous luvisol, calcic cambisol, luvisol, dystic cambisol, podsol and leptosol – WRB nomenclature). A weathering phenotype was determined from quantification of (i) protons and organic acids released in assay solution by bacteria (ii) iron leached from phlogopite lattice into solution by bacteria and (iii) the carbon source consumption (i.e. glucose). These results were then compared to empirical model based on chemical leaching experiments realized in the same conditions in order to simulate the processes involved (4).

This study demonstrated that functional bacterial communities efficiency to weather phlogopite following their origin. Bacterial communities extracted from organic (A) and eluvial (E) horizons were “complexing” bacteria whereas bacterial communities extracted from deeper mineral horizons (B, C) were acidifying. In other words, bacterial communities extracted from A and E horizons produce large amounts of chelating organic acids compared with communities extracted from deeper B and C horizons.

Phlogopite weathering potential of bacterial strains isolated from functional bacterial communities was also determined and quantified. In contrast to bacterial communities, the weathering phenotypes suggest that all isolated strains are “complexing” bacteria (chelate producer). Moreover, no tendencies were observed in function of soil types, organic matter content, rhizospheric effect or leaching of horizons.

Our results suggest that silicate weathering processes by functional bacterial communities depend on soil horizons according to (i) organic matter content, (ii) leaching of horizons and (iii) cationic exchange capacity. Compared with individual bacterial strains, the interaction between strains within communities affect the overall weathering processes, in terms of balance between “complexolysis” / “acidolysis” and also their intensity

Keywords: silicate weathering; organic acids; soil bacteria; phlogopite; nutrient-poor environment


Soil bioremediation of atrazine pesticide by two strains of soil microorganism

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Bioremediation is used in agricultural soils to solve pesticide contamination problems. To maintain soil ecosystems, it is important to avoid the use of foreign microorganisms for soil bioremediation. We used two autochthonous bacteria, *Pseudomonas synxantha* and *Pseudomonas cedrella*, to degrade atrazine pesticide in a previously contaminated soil. These two bacteria had previously been isolated from the same soil. The atrazine degradation in sterile and non-sterile soils was measured by HPLC. Soils were inoculated with both bacteria separately, with their respective non-inoculated controls. After 20 days, more than 20% of the atrazine had been degraded in the sterile soil inoculated with *Pseudomonas cedrella*, and more than 30% in the non-sterile soil. Inoculating sterile and non-sterile soils with *Pseudomonas synxantha* reduced the pesticide half-life by 50 days with respect to the non-inoculated soils. The non-biological degradation of atrazine in sterile non-inoculated soil was 14%. The implementation of bioremediation with selected bacteria contributed to improving atrazine degradation in the studied soil, without altering the soil's microbial ecosystem.

Keywords: Pesticide bioremediation, atrazine, soil microorganisms.

Soil Characteristic Affecting the Mycorrhizal Spore Density in Alluvial Soil of Raniganj Coalfield Areas

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Changes in soil physical, soil organic matter content, soil nutrient concentration, microbial activity and microbial fungal VAM spore density were studied in alluvial soil of different horizon of Raniganj OCP of Eastern Coalfield Limited, India representing about 1-10 m depth of alluvial soil cover over the bedrocks. The characteristics of microbial communities in the humus layer were compared both within different horizon of soil classes and within the pot experiments conducted at the Indian School of Mines, garden campus. During succession or soil genesis processes, the physical and chemical characteristics are correlated with the VAM spore density colonization in different stages of experiments vegetation. The nutrient concentration of the soil OM (organic matter) showed no successional trend on a concentration basis but the C-to-N ratio of organic matter increased with increasing soil age and colonization of Vesicular Arbuscular Mycorrhiza spore density. Thus, the nutrient availability changed during succession. Soil physico-chemical and microbial characteristics increased during the succession changes. The successional decrease in site productivity appeared to be due to leaching of nutrients from the sandy mineral soil and thinning of the humus layer. The present study suggesting the increased importance of VAM mycorrhizal symbiosis for plant performance and increased energy costs among soil microbes in nutrient uptake.

Keywords: Vesicular-arbuscular mycorrhiza, Rhizospheres, Reclamation, Endomycorrhizae; Mining; spores.
Some virulence aspects of *Pseudomonas syringae* pv. *syringae* strains isolated from mango trees

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*Pseudomonas syringae* pv. *syringae* (Pss) is a common inhabitant of a wide variety of plants and the causal agent of bacterial apical necrosis of mango. This plant pathogen have the ability to produce an arsenal of virulence factors which determine the virulence degree of Pss strains. Studies in Pss strains isolated from mango and others plants, showed the ability to produce lipodepsipeptidic toxins, as syringomycin or syringopeptin and mangoxin, an antimetabolite toxin described by our research group. Mangotoxin is a virulence factor produced by a high percentage of Pss strains isolated from mango and it increase the incidence and severity of necrotic symptoms. Furthermore, competition experiments showed that survival values of the wild-type strain were slightly but significantly higher than mangotoxin defective mutants, suggesting that mangotoxin production could also improve the epiphytic fitness. Related with Pss epiphytic fitness has been found in the most of Pss isolated from mango indigenous plasmids with a 62-kb plasmid as the most generalized one. Some studies on this indigenous plasmid showed the relationship between the presence of 62-kb plasmid and copper and ultraviolet light resistance. Copper-resistance in such strains, was evaluated by determining the minimal inhibitory concentration (MIC) of copper sulphate, and UV-resistance by performing survival analysis of Pss cells exposed to different doses of B+A UV-fractions. Both resistance factors were also assayed under field conditions. Molecular analysis by cross-hibridization with specific sequences of copper resistance operon *com* and UV-resistance determinant *mAB* confirmed the presence of homologous genes on 62-kb plasmid. The combination of all theses virulence and epiphytic survival factors present in Pss strains isolated from mango trees, are relevant to understand its lifestyle as epiphytic and pathogenic bacterium.

**Keywords:** virulence factor, *Pseudomonas syringae*, mango, apical necrosis

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Study on the effect of Nitrogen, Glucose and Plant residues on soil microbial C

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The soil microbial biomass is studied as the agent of transformation of both fresh organic inputs to soil and of native soil organic matter itself. Microbial biomass C and organic C were measured in 5 soils selected from 5 long term field cultivation at Bastam area of Shahrood region in Iran. Nitrogen, Glucose and plant residues were used as treatments in a factorial randomized block design with four replications The results were used to discuss the effects of Nitrogen, Glucose and organic C in these soils and the relationships between biomass C and total organic C. This suggests that changes in soil biomass C provide an early indication of changes in total soil organic C following changes in soil management. The dynamics of decomposition and transformation of different substrates (nitrogen, glucose and straw) and the effects of substrate incorporation on the turnover of soil biomass C and the decomposition of soil organic C was studied in 5 soils with different characteristics (e.g. clay content, biomass and organic C contents). From this the mechanisms of priming effects (i.e. accelerated decomposition of soil organic matter following incorporation of substrates) were established. It was also concluded that the measurement of biomass C by fumigation-incubation requires the use of a “control” (unfumigated soil) to estimate the basal respiration (the mineralization of non-biomass organic C) of the fumigated soil during 20, 40 and 60 days of incubations. Soil containing more organic C and receiving larger fresh organic C inputs also have faster rates of soil organic C mineralization, suggesting that the turnover of organic C in such soils is probably faster than in soils containing less organic C and receiving less fresh organic inputs.

**Keywords:** Organic-C, Microbial biomass, Nitrogen, Glucose
Tannin degradation potential and tannase purification from *Enterococcus faecalis*, an isolate from goat faeces

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Tannins, the polyphenolic compounds, have the ability to bind proteins in feed and are reported to inhibit gastrointestinal microorganisms in ruminants; however, tannase activity reported from some gastrointestinal bacteria encouraged to screen the isolates of animal origin for their use either as direct-fed-microbial or for biological detannification of the feeds. Tannases are added as a pre-treatment to tannin-containing animal feed to improve digestibility and to remove the undesirable effects of tannins. The present investigation was carried out to exploit the potential of tannin degrading isolate of animal origin. For this, 36 tannase producing isolates were obtained from rumen and faecal contents of cattle, sheep, goat, wild nilgai and deer. The isolates were identified as *Streptococcus bovis* biotype I (28%), *Enterococcus faecalis* (25%), *S. gallolyticus* (17%), *S. bovis* biotype II.1 (8%) and *Enterobacter sakazakii* (3%). Seven of the isolates were found to possess higher tannase activity and those isolates were further screened for the tannin degraded metabolites in tannic acid rich (1%) broth medium. The chromatographic separation of 24 h culture supernatant showed that all the isolates were able to degrade tannic acid to gallate, further degradation of gallate to pyrogallol was observed only for GF2 (*E. faecalis*) and GF4 (*E. sakazakii*); GF2 was able to further metabolize pyrogallol to resorcinol suggesting its potential to degrade tannic acid to simpler phenolics. Therefore, tannase was purified from the isolate GF2. The enzyme was purified 25-fold after ammonium sulfate precipitation, DEAE-cellulose column chromatography, and Sephadex G-200 gel filtration. The enzyme activity was found between the pH of 5.0-7.0 with an optimum activity at pH of 6.0 and at 40°C. Enzyme activity was found to maximal at 0.25 mM Methyl gallate, further increase in substrate concentration was found to reduce the enzyme activity. The electrophoretic separation of the enzyme preparation indicated it to be made up of a single polypeptide of molecular weight 45 kDa. Further use of the enzyme preparation and isolate are going on to exploit its benefits in terms of its tannin degradation potential in animal feeds rich in tannins.

Keywords Tannic acid, *Enterococcus faecalis*, tannase, purification

Temporal variations in soil fungi communities after biosolarization and its repeated use in pepper crops in Southeast Spain

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The removal of methyl bromide (MB) as a common soil disinfectant in sweet pepper greenhouses in Southeast Spain has led to essay alternatives to it with the minimum environmental effect. A broad spectrum of non-chemical alternatives has been deeply studied for controlling the main pathogens of the crop and keeping acceptable yield levels. Among the different possibilities, the use of organic amendments is recommended by its great number of advantages for soil properties. In this work, biosolarization (BS, biofumigation combined with solarization) disinfectant effects have encouraged to screen the isolates of animal origin for their use either as direct-fed-microbial or for biological detannification. Soil properties. In this work, biosolarization (BS, biofumigation combined with solarization) disinfectant effects have been evaluated in the same way that repeated application of BS treatments had the more its effect making similar to MB. In general, biosolarization treatments had a greater fungal density than MB, but the repeated application of BS showed that the more number of years BS treatments were made in the same soil, the more its effect was making similar to MB.

Keywords: soil fungal communities; manure amendments; biosolarization; pepper

References
Trichoderma harzianum and Aneurinobacillus migulanus in the control of Gladiolus corm rot in a soilless culture system

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Gladiolus corm rot, caused by Fusarium oxysporum f. sp. gladioli is considered one of the most destructive threats to gladiolus production. Aneurinobacillus migulanus and Trichoderma harzianum were tested separately or in combination for the ability to suppress F. oxysporum f. sp. gladioli in soilless culture using Perlite as the substrate. The efficiency of both of antagonists against corm rot was evaluated based on vegetative parameters, rooting parameters and flowering parameters. T. harzianum was more effective than A. migulanus in disease suppression and also enhanced plant growth, increasing flower production and quality. A. migulanus enhanced plant growth when tested alone. The mixture of antagonists reduced the efficiency of T. harzianum. Numbers of T. harzianum CFU in the substrate and on corms increased following application compared with treating with both antagonists. No F. harzianum was detected in the substrate by 120 day after planting, however. A. migulanus CFU significantly decreased on corms when inoculated in combination with T. harzianum and F. oxysporum f. sp. gladioli. However, A. migulanus CFU was not detected in the substrate of the same combination.

SEM suggested that suppressive mechanisms of T. harzianum and A. migulanus differed. T. harzianum appeared to operate through a combination of antibiosis and substrate competition, whereas A. migulanus produced an electron-dense substance which may have inhibited the penetration of host tissues by F. oxysporum f. sp. gladioli. Greater growth of T. harzianum was observed when inoculated alone or with F. oxysporum f. sp. gladioli hypha. It was concluded that T. harzianum provided a more efficient and effective control of F. oxysporum f. sp. gladioli corm rot of Gladiolus when inoculated without A. migulanus.

Keywords: Gladiolus; Trichoderma harzianum; Aneurinobacillus migulanus; Soilless culture; CFU.

The importance of the biomicroworld on macroproduction

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Like animals, plants in natural environments are colonized by several types of microorganisms. Some may increase plant resistance to biotic and abiotic stress. Bio fertilizers are usually formulated based on soil microorganisms (fungi and bacteria) associated with the rhizosphere that promote and benefit plant nutrition. The mechanisms involved in the relationship may vary, and the effects may be synergistic between distinct microbial functional groups (mycorrhiza, phosphorous solubilizers, and nitrogen fixers). Bio fertilizers are widely used in biological agriculture as an alternative to less sustainable chemical fertilizer inputs used in intensive agriculture. This work attempts to combine chemical and biofertilizers in order to increase plant productivity taking advantage of plant microbiota interactions in a system not limited by macro-nutrient availability, and increasing system sustainability.

Several field trials have been done in Portugal using chemical fertilizers (P, NP or NPK) coated with microorganisms. It has been observed that, independently of the soil type, and depending on the plant culture, coated fertilizers may improve yield by 5-18% relative to non-coated fertilizer. The aim of this work is to deepen knowledge of the mechanisms associated with this increased plant productivity in the presence of fertilizers coated with microorganisms.

Maize (Zea mays, cultivar Moncada) plants were grown under greenhouse conditions in pots (7 L capacity, 6 kg soil, 1 plant per pot) with non-humic litolitic soil from a maize field (Montemor-o-novo, Portugal), from April to May. Fertilizers were applied in concentrations similar to those used by farmers. Plants were allowed to grow until the development of the flag leaf. Immediately before collecting the plants, the reflectometry of each plant was recorded (UniSpec, PP Systems) and used to determine the water index (WI). Shoots and roots were collected separately. Leaf dry weight was determined after drying the plant material at 70°C until constant weight. Leaf biomass was used to analyse the mineral content and the natural nitrogen isotope signature. Soil from the rhizosphere was sampled to assess the physiological profile of the bacterial community (Biolog), the abundance of fungi and bacteria (CFU), the similarity of the bacterial community among the distinct treatments, arbuscular mycorrhizal colonization and phosphatase activity.

Results showed that microorganisms can affect plant biomass accumulation through several distinct mechanisms: increased phosphorous and nitrogen availability and higher water use efficiency. It was shown that the source of phosphorous used as a fertilizer was a main component of the micro-organisms plant interaction. When insoluble forms of phosphorous were used, root AM colonization was stimulated, unless the fertilizers were coated with microorganisms efficient in soil solubilization, in which case root AM colonization significantly decreased. It was also detected that when nitrogen in the soil was limiting to plant growth, plants fertilized with coated and non-coated fertilizers used distinct main sources of nitrogen. Finally, results indicate improved water use efficiency in the presence of coated fertilizers.

Together, results show that the use of fertilizers coated with microorganisms had an effect on the structure and function of soil, which was reflected in plant productivity. Results are interpreted as an evidence for the benefits of coating fertilizers with microorganisms as a compromise between the advantages of the “biological” and “intensive” systems of production.
The role of fungi in the decomposition processes in forest soils

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Hardwood and coniferous forests represent one of the most important biomes in temperate zone. Forest soils accumulate significant amounts of carbon deposited annually through litterfall and the accumulation is a prerequisite for the formation of soils with distinct litter and humus horizons. In hardwood forest soils, saprotrophic basidiomycetes and ascomycetes dominate the litter horizon while ectomycorrhizal species dominate in deeper soil [1]. There is a sharp gradient of soil physical and biological properties including the amount of microbial biomass, organic carbon, humic material, soil respiration and the activity of extracellular enzymes participating in carbon transformation [2]. All of these parameters decrease with soil depth reflecting the availability of nutrients. The presence of saprotrophic basidiomycetes in soils results in an increased activity of the lignonolytic enzymes Mn-peroxidase and laccase, polysaccharide hydrolases and chitinase. Isolated strains of saprotrophic cord-forming basidiomycetes (e.g. *Hypholoma*, *Rhodocollybia*, and *Gymnopus*, spp.) are able to mineralize significant part of lignin contained within litter and to form humic substances from lignin and other soil components [3]. In a farther, slower step, the same fungi continue in the mineralization of humic compounds. *In vitro* experiments demonstrated that Mn-peroxidase is the required enzyme for humic acid and lignin transformation by basidiomycetes while there is no contribution of laccase to this process. The potential to degrade and transform lignocellulose is, however, limited *in situ* when basidiomycetes interact with other members of soil biota. In the case of lignocellulose transformation, the competitors are mainly nonbasidiomycetous microfungi and certain soil bacteria, e.g. the *Actinomyces*. Their contribution to litter transformation is obvious from the analyses of microbial community composition of litter in different stages of decay and by the fact that the fine chemical properties revealed by pyrolysis and polysaccharide analysis show a distinction between the biomass transformed by basidiomycetes alone and by the whole soil microbial community [3]. The studies on opportunistic micromycetes from forest soils showed that simple carbon compounds are the most widely used substrate while they are unable to attack lignin and their abilities to decompose cellulose are limited. The decomposition of litter seems to be at least partly regulated by the chemical composition: the availability of NH3 increases litter decomposition while high P content decreases lignin removal. The rate defining step for fungal litter decomposition is cellulose hydrolysis since the loss of litter mass closely correlates with the chemical composition of forest soils. Functional differences among the members of soil fungal communities are reflected in a vertical stratification of soil fungal communities where saprotrophic species dominate the upper horizons and actomycorrhizal fungi the deeper horizons, but also in the differences of vertical distribution of fungi involved in polysaccharide decomposition in individual soil horizons as demonstrated using the 13C-stable isotope probing.


Keywords basidiomycetes; enzyme; forest microbiology; lignocellulose degradation; microbial ecology; soil fungi

The role of plant growth promotion rhizobacteria on sustainable field crop production

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During the past decades the increasing use of fertilizers and highly productive systems have created environmental problems such as deterioration of soil quality, contamination of surface and groundwater, as well as air pollution, reduced biodiversity, and suppressed ecosystem function. Bacteria that stimulate plant growth are usually referred as Plant Growth Promoting Rhizobacteria. These bacteria can be affected by a wide range of factors including plant type and age, distance from the soil to the root, soil characteristics, and agronomic practices. These bacteria vary in their mechanism of plant growth promotion but generally influence growth via P solubilization, nutrient uptake enhancement; phytohormone, Antibiotics and Sidrophores production, Nitrogen fixation, reduce environmental stress and Induced systemic resistance. This paper try to explain factors affect on bacterial efficiency and mechanisms that effects on plant growth as mentioned above also are reviewed.

Keywords plant growth promotion rhizobacteria (PGPR); crop production; beneficial effects
The toxicity and histopathology of Bacillus thuringiensis Cry1Ba toxin to Spodoptera frugiperda (Lepidoptera, Noctuidae)

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Bacillus thuringiensis (Bt) synthesizes entomopathogenic protoxins, in soluble and activated forms bind to a membrane receptor and therefore lyses midgut epithelial cells. Bt protoxins are codified by different cry genes, where Cry1 proteins are well known for their high toxicity against lepidopterans and dipterans insects. In this study, the insecticidal activity and the histopathological effects of bacterial suspension and purified Cry1B protein from Bt thuringiensis strain 4412 in the midgut epithelial cells of S. frugiperda larvae.

The Bt strain utilized in this study, which codifies the protein Cry1Ba was supplied by the International Entomopathogenic Bacillus Center (Institute Pasteur, Paris). For the bioassays with bacteriological suspension the strain was grown in Usual Gelled Medium at 28°C and 180rpm for 48 h. After that, the cultures were centrifuged at 4,500 rpm for 15min and the supernant was discarded. The bacterial pellet was recovered with sterile distilled water. The bacterial concentration was determined with Neubauer chamber technique and optical microscopy. Second instar larvae of S. frugiperda were obtained from colonies reared in the insect’s chamber, maintained at 25 ± 2°C, 80% Relative Humidity (RH) and 12 h photoperiod. In vivo assays, with S. frugiperda were realized in Biological Oxygen Demand (B.O.D.) chambers, at 25 ± 2°C, 80% RH and 12h of photoperiod. The culture, corresponding to 1×10⁶ cells/mL, was applied to the Poitout diet, previously conditioned in mini-plates (30mm diameter), where larvae were individualized. In the control group, the culture was substituted by sterile distilled water. Twenty larvae were used for each treatment. The mortality was observed until de 7th day after applying the treatment and all bioassay data were corrected by Abbott’s formula. The toxicity of Bt thuringiensis 4412 strain was also determined by the Medium Lethal Concentration (LC₅₀), using purified protein through sucrose gradient (67 to 88%). The insects were individualized, as already mentioned, and the Poitout diet was substituted by disks of fresh corn leaf, where the protein was applied in the concentrations of 2.0, 6.0, 18.0, 54.0 and 162,0 µg/mL. In the control group the protein was replaced by sterile distilled water. Twenty insects were evaluated and each treatment was replicated three times, totaling 90 insects on treatment. The histopathology evaluations of B. thuringiensis thuringiensis 4412 bacterial suspension and its Cry1Ba purified protein were realized in S. frugiperda larvae of 2nd instar, in which the mortality was evaluated daily. In addition a kinetic experiment was ranne with collected larvae in time of 1, 3, 6, 12 and 24 hours after the treatments application.

The tested strain, B. thuringiensis 4412 (Cry1Ba), was highly toxic in the bioassays with 100% of mortality to S. frugiperda larvae and was submitted to protein purification and determination of LC₅₀. The results indicated that the expressed Cry1Ba protein was highly toxic to S. frugiperda, with a LC₅₀ of 10.85 µg/mL. The histopathological analysis of S. frugiperda midgut treated with B. thuringiensis thuringiensis 4412 bacterial suspension and Cry1Ba protein, showed a progressive loss of epithelial cell definition from 3h onward, in both treatments. At 24h post treatment, the majority of larvae treated with bacterial suspension were already dead. Their midgut changes, observed under the light microscopy, included vacuolization of the cytoplasm, hyper trophy of the epithelial cells, and vesicle formation in the apical region of both goblet and columnar cells. Also, the brush border membrane was damaged, especially in goblet cells. The larvae treated with Cry1Ba purified protein, in despite of not dying as fast as in the bacterial suspension treatment, also showed severe damage in the midgut epithelial cells. These injuries included degeneration of the epithelium and consecutive lyses and leakage of cytoplasm material in the lumen, showing debris and disrupted cells.

In conclusion, the present investigation provided evidence that both bacterial suspension and Cry1Ba purified protein of B. thuringiensis thuringiensis 4412, have a strong insecticidal activity against S. frugiperda larvae which was observed by the degeneration of their midgut epithelium.

Keywords: Cry toxin, Bacillus thuringiensis, Lepidoptera, Bioassays.
An assessment of the microbial diversity present in water from three Parisian surface water treatment plants

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The microbiological quality of drinking water is currently assessed using culture-based methods, even though plate count techniques are known to significantly underestimate the total number of bacteria any in given environment. Any remaining organisms present after treatment in finished drinking water are then released into the distribution system and may interact with microbial populations present in the water distribution network, and can be involved in biofilm growth, nitrification, microbial-mediated corrosion and pathogen persistence. As a consequence, knowledge of the microbial ecology of drinking water treatment plants is of prime concern for drinking water producers.

In order to assess both the bacterial and eukaryotic diversity in drinking water produced from three Parisian surface water treatment plants, we utilized an rDNA based approach to overcome cultivation-based limitations. We used the serial analysis of V6 ribosomal sequence tag (SARST-V6) method to examine the bacterial diversity in finished chlorinated drinking water and observed a considerable degree of diversity. The taxonomic composition of the microbial communities was found to be dominated by members of the phylum Proteobacteria. Additionally, a large proportion of sequences were found to be distinctly related to other database sequences and their presence and phylogeny were confirmed by a full-length 16S rDNA analysis. Notwithstanding the potential under-representation of certain bacterial phyla using the SARST-V6 primer pairs, as revealed by a refined computer algorithm, our results suggest that 16S rDNA corresponding to a variety of bacterial groups can be detected in finished drinking water [1]. An assessment of eukaryotic 18S rDNA diversity present in finished drinking water samples was also performed. The 18S rDNA sequences affiliated to the Amoebozoa, Ciliophora and Metazoa lineages were found to be the most abundant phylotypes observed in the drinking water samples, showing that finished drinking water can also contain 18S rDNA sequences representing a variety of eukaryotic taxa [2].

To ensure the microbial quality of drinking water from treatment plant to consumer tap, a final treatment step of disinfection is performed in order to reduce the number of bacteria to an acceptable level in the processed water and to limit microbial growth in the drinking water distribution system. In order to investigate the consequences of the disinfection step, we examined the variations of bacterial diversity prior to and after chlorine disinfection of drinking water prior to its entry into the distribution network. For this purpose, the bacterial diversity present in treated water was studied after GAC filtration and final disinfection from two surface water treatment plants supplying the city of Paris (France). Through the use of 16S rDNA clone library construction, the bacterial abundance patterns and taxa evenness were found to be different between samples, suggesting that the disinfection step markedly affects the bacterial community structure present in GAC water. Our results indicate that certain bacterial groups are particularly affected following the chlorine-based disinfection treatment performed in the two DWTPs, suggesting different levels of sensitivity to the disinfection treatment.

As a consequence, the data and approaches presented in these studies can be useful to elucidate the complexity and dynamics of the bacterial populations in drinking water treatment plants. Clearly, further research is required to fully identify and characterize the microbial ecology of drinking water treatment plants and their effects on the water distribution network. This will, in turn, improve our understanding of the potential risks associated with the bacterial groups present in drinking water production and distribution networks.

Keywords: Finished drinking water, chlorine disinfection, 16S and 18S rDNA, phylogenetic analysis, microbial diversity.


Antibacterial activity of extracts from different Origanum vulgare clones grown in Latvia.

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The use of plant compounds for pharmaceutical and antimicrobial purposes is well-known. Origanum vulgare is widely used in pharmacies and folk medicine. Origanum vulgare clones grown in Latvia differ from their phytochemical content. The aim of the study was to determine the antimicrobial activity of these clones. The antibacterial activity was assessed against bacteria (Escherichia coli, Pseudomonas aeruginosa, Staphylococcus epidermidis, Bacillus cereus) and yeast (Candida albicans). A sensitivity tests were performed in the liquid nutrient media for bacteria and liquid malt media for Candida albicans. Plant ethanol extracts from leaves and flowers (25-40 g of fresh matter per L) were added to the growth media. Extract and media proportion was 1:20. Microorganisms growth were detected spectrophotometricaly at wavelength 550 nm after 24 and 72 hours of incubation at 28 °C. Origanum vulgare leaves and flowers showed different activity. The antimicrobial activity depends on O. vulgare clone. Different clones showed unlike activity on used microorganisms and it depends on oregano chemical content. Antimicrobial activity decreases during isailisation.

Keywords: Origanum vulgare, antimicrobial activity.
Antifungal potential of *Cladosporium cladosporioides* (Fres) De Vries metabolites in reduction of coffee contamination by toxigenic Aspergillus genera

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Brazil is the biggest world coffee producer and exporter and the second world consumer. The relationship between microorganism occurrence and bad coffee beverage quality has been object of several researches. It must be considered yet the risk represented by mycotoxins producer potentials by fungi in coffee as ocratoxin A whose mainly produce yeasts of fungi genera. Researchs developed showed association of coffee fruits and Cladosporium genera that is always related with good coffee quality. This fungus was collected and identified as *Cladosporium cladosporioides* (Fres) De Vries. Fungus isolates were tested and selected according to its biodeterioration properties in relation sensory and safe coffee fruits characteristics. The present research showed the extract capacity obtained from a selected isolate of *Cladosporium cladosporioides* (Fres) De Vries, obtained after testing four extracts in the sporulation and germination of *Aspergillus ochraceus* Wilhelm and *Aspergillus niger* van Tieghem fungi obtained from contaminated coffee beans. It was observed that extract inhibited the sporulation in 75,5% (methanol extractor) and 51,1% (methanol extractor) and spore germination in 75,0% (Dimethyl Sulfoxide - DMSO extractor) and 83,0% (Dimethyl Sulfoxide - DMSO extractor) of *A. ochraceus* and *A. niger* respectively. The conclusion is that metabolites produced by the fungus are efficient in important fungi phase development reduction in coffee contamination, and consequently by OTA. We must stand out that the importance of the fungus natural preservation on field or its introduction in the coffee plantations where it doesn’t occur or was eradicated by inadequate plantation management. The development of a natural fungicide based in the fungus metabolites is subject of a research that is in development at this moment.

Antiocotic potency of *Drosera intermedia* extracts on fungi and yeasts causing biodeterioration on food commodities

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Fungi, may have a great impact in our life as pathogens, in food degradation or in toxin production [1]. For example, *Zygosaccharomyces bailii* is one of the most dangerous spoilage yeast in wine and soft drink industry and *Aspergillus fumigatus* has become the most prevalent airborne fungal pathogen, causing severe and usually fatal invasive infections in developed countries. Some fungal species secrete toxic secondary metabolites, known as mycotoxins, which can spoil foods and commodities worldwide. When ingested, inhaled or absorbed through the skin, mycotoxins will cause lowered performance, sickness or death on humans and animals [2]. The present work reports the inhibitory activity of *Drosera intermedia* (H.) extracts against four food spoilage yeasts (*Zygosaccharomyces bailii* PYCC 4806, *Saccharomyces cerevisiae* PYCC 4072, *Debaryomyces hansenii* PYCC 2968 and *Pichia membranaefaciens* PYCC 2489) and seven fungi strains (*Aspergillus fumigatus* MUM 98.02, *Aspergillus fumigatus*, *Aspergillus niger* MUM 03.43, *Aspergillus niger*, *Aspergillus parasiticus* MUM 92.02, *Aspergillus flavus* MUM 92.01 and *Penicillium expansum* MUM 02.03) responsible for food deterioration and associated to mycotoxin production. *D. intermedia* is an insectivorous plant species that is becoming increasingly scarce and has been produced by micropropagation for the purpose of this work [3]. Several *D. intermedia* extracts (water, methanol and hexane) were tested against yeast and fungal strains using the agar disc diffusion assay followed by the determination of minimum inhibitory concentrations (MIC). The hexane extract showed a broad activity spectrum against all tested microorganisms, followed, in activity, by the methanol and water extracts. The two *A. fumigatus* strains were the most susceptible, scoring MIC values of 15.63 μg/ml, 250 μg/ml and 2000 μg/ml against the hexane, methanol and water extracts, respectively. The most susceptible yeast strain to the *D. intermedia* extracts was *Z. bailii* PYCC 4806 for which MIC values of 7.81 μg/ml, 125 μg/ml and >1000 μg/ml were obtained for hexane, methanol and water extracts, respectively. LC-MS and NMR analysis of the hexane extract showed that its major compound was the naphthoquinone plumbagin [4]. A further purified extract was used for the MIC assays which afforded increased activities. The MIC values obtained for all tested microorganisms decreased significantly, for *A. fumigatus* MUM 98.02 and *Z. bailii* PYCC 4806 MIC values of 0.08 μg/ml and 1.95 μg/ml were scored, for instance. These results suggest that *D. intermedia* is a source of interesting biocompounds for the food industry and that plumbagin might have antifungal potential.

**Keywords** mycotoxins; food safety; MIC; plumbagin


Acknowledgements: Tomás Grevenstuk and Sandra Gonçalves acknowledge a grant from the Portuguese Science and Technology Foundation (FCT, Grant SFHR/BD/31777/2006 and SFHR/BPD/31534/2006, respectively).
Antimicrobial susceptibility of Campylobacter jejuni isolated from poultry products and human cases of campylobacteriosis

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Members of genus Campylobacter are well known as leading causes of bacterial gastroenteritis in humans. In general, campylobacteriosis is resolved without antimicrobial intervention; however, treatment is crucial to manage severe or invasive illness. The antimicrobial resistance of Campylobacter spp. has been increasing worldwide. In the present study, several strains of Campylobacter jejuni (n=159) collected from poultry food products (61%) and from human cases of campylobacteriosis occurred in Portugal (39%) were evaluated concerning their antibiotic susceptibility. The MICs of the tested antibiotics were determined by the agar dilution test according to the CLSI instructions (2009). Enterococcus faecalis ATCC29212 and Staphylococcus aureus ATCC29213 were used as controls.

0.6%, 5.6%, 30%, 60%, 74% and 80% of the isolates were resistant to chloramphenicol, gentamicin, erythromycin, tetracycline, nalidixic acid and ciprofloxacin, respectively.

Globally, the antimicrobial susceptibility to antibiotics is related with the origin of the strains. In fact, and for all the tested antibiotics, the MIC50 of the food isolates was higher than the MIC50 of the clinical ones. These differences in the antibiotic susceptibility between food and clinical C. jejuni isolates might be related with the antibiotic drugs that are commonly used in animal production. These resistant bacteria can be transmitted to humans and affect the human health.

Keywords Campylobacter jejuni; antibiotic; Susceptibility

Antimicrobial activity of rhamnolipids from P. aeruginosa PA01 against L. monocytogenes

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Introduction. The biosurfactants properties are of interest to a wide range of industrial fields, from petroleum to pharmaceuticals. In food industry, these microbial compounds exhibit useful properties as emulsifiers, anti-adhesive and antimicrobial agents. Listeria monocytogenes is an important foodborne pathogen and it’s known that many strains are able to develop biofilms in a variety of surfaces. The aim of this work was to evaluate if purified rhamnolipids presents potential to inhibit L. monocytogenes planktonic and adhered cells growth.

Material and Methods. The biosurfactants from P. aeruginosa PA01 were previously produced and purified. The Minimal Inhibitory Concentration (MIC) of rhamnolipids was evaluated using the microbroth dilution technique. A microtiter plate containing TSYE broth was inoculated with a 10^6 CFU/mL culture in the presence of the biosurfactant at different concentrations of 40 mcg/mL to 3600 mcg/mL and incubated for 24 hours at 35°C. The growth was evaluated by reading the absorbance (590 nm) against control. Aiming to evaluate if the presence of the biosurfactant would influence the biofilm formation, a bacterial suspension was prepared to attain 10^9 CFU/mL and 20 μl of this suspension was inoculated in each well containing the broth and the same concentrations of biosurfactant used in the MIC assay, the microtiter plates were then incubated at 35°C for 24 hours. The wells were washed with water, fixed for 15 min with methanol and stained for 20 min with crystal violet 1% (w/v). After washing with water, the stain was extracted from the biofilms using glacial acetic acid (33%). The optical density (570 nm) of this stained solution was used to measure the adhered cells. The tested microorganisms were two strains of L. monocytogenes (ATCC 19112 and ATCC 7644) which have great ability to form biofilms.

Results. The results showed L. monocytogenes had its planktonic and adhered cells growth inhibited by the rhamnolipids at different concentrations (ranging from 40 μg/mL up to 3600 μg/mL) comparing to uninoculated control. The rhamnolipids were able to inhibit L. monocytogenes ATCC 7644 growth showing a MIC of 40 μg/mL. The adhered cells growth was inhibited about 97% with the purified rhamnolipids at the different concentrations. A similar profile was observed to L. monocytogenes ATCC 19112 strain, which demonstrated a MIC of 40 μg/mL of rhamnolipids, and an increase inhibition reaching 98% with 3600 μg/mL. The adhered cells growth was inhibited at about 95% for all biosurfactant concentrations.

Conclusion. These results demonstrated that rhamnolipids obtained from P. aeruginosa PA1 have excellent antimicrobial potential against L. monocytogenes strains.

Keywords microbial growth; adhesion; crude rhamnolipids, purified rhamnolipids; antimicrobial activity

Acknowledgements to CNPq for scholarship.
Antimicrobial and physical and mechanical properties of composite whey protein and starch edible films

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In this study antimicrobial properties of composite films from whey protein and starch containing 0.5%, 1% and 1.5% v/v of ziziphora and satureja essential oil against salmonella enteritidis, listeria monocytogenes and penicillium roqueforti were investigated. In order to improve the barrier properties of films against water vapor transmission, oleic acid was incorporated to edible films. Physical and mechanical properties of edible films including thickness, moisture content, water vapor transmission rate, percent elongation at break, tensile strength and elastic modulus were measured.

Films containing ziziphora essential oil showed larger inhibitory effect. Films were more effective against Gram-positive bacteria than Gram-negative bacteria and don’t have more effect on mold.

By addition of oleic acid thickness and percent elongation at break of edible films were increased and the other properties were decreased significantly. Physical and mechanical properties changed by incorporation of essential oils.

Results of this study suggested that antimicrobial activity of some essential oils in whey protein edible film was obvious.

Keywords: Edible film, whey protein, starch, ziziphora, satureja, Oleic acid, Gram-positive bacteria, Gram-negative bacteria, Antimicrobial properties, Physical properties, Mechanical properties.
Application of Quantitative RT-PCR in expression study of the ammonium and hexose transporters during the rehydration of *Saccharomyces cerevisiae* in active dried form

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The use of selected yeasts in the form of active dry yeast (ADY) in winemaking implies a short reactivation period in aqueous media. The rehydration restores the active metabolic condition that is necessary to face the fermentation with good fermentative and competitive abilities. Despite the importance of this phase, to date, there have been a small number of reports on the events that occur during rehydration.

In this work we report the application of the quantitative RT PCR (RT qPCR) to study gene expression during the rehydration using a relative quantification method. The goal is to observe transcriptional modifications in some genes codifying for ammonium and hexose transporters during the rehydration process and to determine whether their expression could be modulated by altering the composition of the rehydration medium.

First, we evaluated the expression stability of eight potential reference genes using three statistical methods: BestKeeper, GeNorm and the method proposed by De Kok. Generally, using these three methods, more stable genes included *18S, ACT1, QCR9,* and *LSC2* but using the GeNorm classification, *LSC2* was classified in the last positions. To select the best genes, an arbitrary score was attributed to each gene for each statistical calculation. Based on this ranking, *18S, ACT1, QCR9* provided the best results.

This reference system was then applied to study the expression levels of the ammonium transporters *MEP1, MEP2,* and *MEP3* and the hexose carriers *HXT2, HXT3* and *HXT6/7.*

Regarding the ammonium transporters tested, the results demonstrated that the yeast immediately reacted to rehydration only when a fermentable carbon source was present in the medium. Furthermore, *MEP2* expression was modulated by the ammonium concentration, indicating that nitrogen catabolite repression (NCR) is active during the rehydration phase. The rehydration in water, frequently used by wine producers, doesn’t modify the transcriptional pattern of dry yeast. These results were confirmed by the data relative to the ammonium assimilation.

The study of the expression of the hexose carrier shows that the sugar assimilation system is fully active and it is responsible for the sugar uptake in the cell after few minutes of rehydration. It was observed that the substrate affinity is not the only mechanism that modulates the expression of the hexose carriers but other unknown regulation factors influence the transcription of some of them.

New information on expression changes during rehydration could lead to improvements in this step of winemaking by changes in the medium composition, temperature, rehydration time, and other factors. This could lead to competitive advantage for the inoculated strains and contribute to successful vinification.

Keywords: RT qPCR, ADY, *Saccharomyces cerevisiae*

Aspects of the regulatory mechanisms in the Alkali-Tolerance Response (AITR) in *Listeria monocytogenes*

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*Listeria monocytogenes* can survive and grow in relatively high pH environments, giving it a significant competitive advantage in the transient alkali conditions environments, which can occur during decontamination of food processing systems and the human gastrointestinal system. *L. monocytogenes* is able to launch a significant adaptive response to alkali stress, the Alkaline-Tolerance Response (AlTR), enabling it to [a] withstand alkali stress and/or become resistant (stress hardened) to higher levels of such stresses, and [b] be cross protected against unrelated stresses, such as osmotic or ethanol stress. Studies using *sigB* isogenic null mutants have established an important role for the alternative sigma B factor in such alkali tolerance in *L. monocytogenes*. Scanning Electron Microscopy, hydrophobicity assay and gas chromatography studies have established that alkali adaptation(s) in *L. monocytogenes* involve changes in cell morphology, cell surface and in the proportions of cellular membrane fatty acids. DNA microarrays and 2D-gel electrophoresis have demonstrated that alkali responses involve a complex network of changes in gene and protein expression. There is a clear distinction between the mechanisms involved in short and longer term alkali stress responses, and clear differences between resistance mechanisms at different sublethal pH values e.g. (pHs 9.0 and 9.5). Microarray studies established that AITR involves activation of processes which cause an intracellular accumulation of protons, including metabolic changes leading to increased acid production, activation of ionic pumps (mainly Na+/H+ antiporters) and the production of alkali specific (i.e. aminases) and general stress proteins. The improved understanding of the AITR gained in this study contributes to a wider understanding of the pH homeostasis of *L. monocytogenes* and should underpin the development of better methods for the control of this significant pathogen.

Keywords: alkali stress, *Listeria monocytogenes*, microarrays
β- Glucans Production and Manoproteins Release in Yeasts

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It is largely known that in Enology, the contact between wine and lees after alcoholic fermentation, offers to final product some good characteristics that consumers can appreciate. Polysaccharide and manoprotein are the most important compounds liberated coming from yeast cellular walls. This process is, indirectly link, to beta glucanasa activity. Autolysis capability of yeast and production of 1, 3 beta endoglucanase were studied in the present work, and therefore, the liberation of polysaccharide / manoprotein into a culture medium that simulated fermentation conditions. 23 Saccharomyces strains isolated from spontaneous fermentations in different wineries were use for this study. The most important conditions for beta glucanase enzyme production and time of growth in appropriate culture medium were optimized. For each culture yeast, the enzymatic activity and polysaccharide liberation were quantified, both in supernatant and cellular extract.

Results show that there is not relationship between enzymatic activity and polysaccharide liberation. On the other hand, all yeasts were significantly different regarding to polysaccharide liberation, in the two different fractions. These results offer for next works, the possibility of studying the effect of cellular extracts on quality wine.

Keywords: Yeast, manoprotein, enzyme.

Bacteriocin production by Lactobacillus ssp. V69 and some aspects of its mode of action against Listeria monocytogenes ScottA

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Lactic acid bacteria are widely used as starter cultures and play an important role in food preservation, microbiological stability and production of aroma compounds in various food products. Many of these lactic acid bacteria produce bacteriocins. By definition, bacteriocins are small proteins with bactericidal or bacteriostatic activity against genetically closely related species. Their mode of action may include pore formation in the cell wall, degradation of cellular DNA, disruption through specific cleavage of 16S rDNA, and inhibition of peptidoglycan synthesis. Strain Lactobacillus spp. V69 was isolated from charqui, a traditional salted and dried fermented meat product from Brazil. Despite the low Aw in charqui, halophilic microorganisms can grow and cause spoilage. The growth of these undesired bacteria can be inhibited by antimicrobial components produced by microorganisms that are part of the natural microflora of the product.

Strain Lactobacillus spp. V69 produces an antibacterial substance (bacteriocin) active against L. monocytogenes ScottA in MRS broth at 30°C or 37°C. The bacteriocin showed to be inhibitory to different serotypes of L. innocua and L. monocytogenes, besides Lactobacillus sakei, Staphylococcus aureus and halophilic bacteria isolated from charqui. Antimicrobial activity complete inactivation was observed after treatment of the cell-free supernatant with Proteinase type XIV, pointing the proteonaceus nature of the antimicrobial agent. No change in activity was recorded when treated with catalase or α-amylase. The stability of the bacteriocin was not affected by the presence of 1% SDS, Tween 20, Tween 80, Urea, EDTA or NaCl and remained stable after incubation for 2 h at pH from 2.0 up to 12.0. Stability of the bacteriocin was recorded after 120 min at 25, 30, 45, 60 or 100°C. Different levels of absorption were recorded when bacteriocin V69 was in contact with Listeria monocytogenes ScottA, Lactobacillus sakei ATCC 15521 and Enterococcus faecium ATCC 19443. This absorption was dependent of temperature (tested 4, 25, 30 and 37°C), pH (tested 4, 6, 8, 10) and the presence of selected chemicals (NaCl, tween, glycerol and SDS). The highest adsorption to Listeria monocytogenes Scott A was recorded at 4°C, pH 2.0 and 4.0 and in presence of NaCl. These results are important knowledge in order of application of this bacteriocin in the food biopreservation.

Addition of bacteriocin V69 to a 3-h-old and 7-h-old culture of L. monocytogenes ScottA were recorded after 25h of treatment, suggesting that the mode of action of bacteriocin V69 is bactericidal.

Keywords: bacteriocin, Lactobacillus spp., anti-Listeria activity, halophilic bacteria.
Bacteriophage contamination and fermentation of *Natto* by *Bacillus subtilis* (natto) – Study of phage related key enzyme that spoils sticky texture of natto-

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The *natto*, a traditional Japanese soybean food fermented by *Bacillus subtilis* (natto), is occasionally spoiled by bacteriophage contamination. The spoilage can be seen even when the number of the contaminated phage is very small and *B. subtilis* (natto) grows normally. We isolated a bacteriophage PhNIT1 and identified a key enzyme (PghP) produced by the phage that hydrolyzes poly-gamma-glutamate (PGA). PGA is a polymer of glutamate linked by gamma-glutamyl peptide bond (degree of polymerization (DP) > 10,000). It is a viscous material and provides sticky texture to the *natto*. The sticky texture is accepted as an essential quality of the *natto*. The enzymic degradation of PGA dramatically reduces the DP and the viscosity, and damages the *natto* products. We examined the degradation of PGA by PghP in detail. PghP hydrolyzes PGA by endo-type manner to penta-, tetra-, and tri-, oligo-peptide. PghP requires Zn ion for the activity and is inactivated by a Cys-modifying substance moniodoacetate. The pghP gene encoded by the phage genome was cloned and the nucleotide sequence was determined. However, deduced amino acid sequence has no similarity to known peptidases, which makes molecular level elucidation of the catalysis difficult. Recently, 3D structure of PghP was determined and it revealed that the tertiary structure of PghP, unexpectedly, was homologous to that of bovine carboxypeptidase A or putative N-formylglutamate amidohydrolase of *Rastonia eutropha* and that PghP belonged to M14 metallo peptidase family. Catalytic residues (His-Glu-His) and Zn binding motif were assigned. The α/β open sheet structure is conserved well between PghP and phylogenetically very far enzymes in spite of the diversity in amino acid sequence.

Keywords: Fermented soybean, Bacteriophage, *Bacillus subtilis* (natto), poly-gamma-glutamate, hydrolyase, natto.

Bee pollen-containing culture media can stimulate production of patulin by *Penicillium expansum*

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Patulin (PAT) is a mycotoxin that occurs worldwide in apple and apple products. PAT is produced by several fungal species, but mainly by *Penicillium expansum*. These molds are common post-harvest pathogens of apples and pears. Fruits decayed by these molds are also likely to be contaminated with PAT, which is heat stable, resists processing, and has been found in apple products. It has been reported that PAT is genotoxic and causes damage to DNA or chromosomes.

It is usually accepted that mycotoxin production is dependent on various factors, such as the strain, the substrate and environmental conditions. Patulin-producing species are widely distributed but only a percentage of the strains belonging to reported producing species are PAT producers. This percentage may increase when studies in progress can establish the ideal conditions for PAT biosynthesis. The correct characterization of patulin-producing strains is necessary to correctly assess their occurrence in contaminated substrates, the suitable characterization of chemotypes and for other studies, especially DNA-based studies.

Bee pollen is a basic food for bee larvae development due to its high protein content. This food contains all the essential amino acids, minerals, vitamins, enzymes, growth regulators, fatty and organic acids, flavonoids, lipids, sterols and certain carbohydrates. Beekeepers catch pollen in traps put at the hive entry. Pollen remains in traps for some time. Afterwards, it is taken out and carried to stores where it is cleaned, dried, sometimes fumigated, stored and marketed. Before harvest or during these stages pollen may be contaminated by several fungal species.

The aim of the present study was to assess the capacity of bee pollen as a substrate for production of PAT by strains of *P. expansum*. The solid media assayed were Yeast Extract Sucrose medium (YES), YES supplemented with 0.5, 1, and 3% bee pollen, Potato-glucose medium, Wickerham medium, Aflatoxin Production medium and Bee Pollen-glucose medium at different concentrations of bee pollen (0.5, 1 and 3%). Cultures were kept at 25 °C for 2 weeks and analyzed for PAT by LC with UV detection on days 3, 7, and 14.

PAT production in media containing bee pollen was significantly higher than production in the other culture media regardless of incubation time. A correlation between the proportion of pollen added to medium and PAT level was observed. On the basis of the preliminary results obtained in this study it can be hypothesized that bee pollen consumption may constitute an important risk factor concerning the presence of PAT in the diet of consumers of that nutritious food.

Keywords: bee pollen; patulin, *Penicillium expansum*

Acknowledgements: the authors wish to thank financial support from FEDER and Spanish Government “Ministerio de Ciencia e Innovación” (Project AGL2007-66416-C05–01/ALL, and two research grants).
Behavior of shiga-toxin-producing *Escherichia coli* (STEC) of serotype O113:H21 to front pH, water activity, time and temperature.

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*Escherichia coli* strains are part of the intestinal microbiota of humans and most warm-blooded animals. However, several pathotypes are recognized and involved in intestinal disease, including enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC) and Shiga toxin-producing *E. coli* (STEC/EHEC). STEC is involved in severe human diseases such as bloody diarrhea, hemorrhagic colitis (HC), and hemolytic uremic syndrome (HUS). Two subgroups to STEC are described based on the presence of a pathogenicty island called Locus of Enterocyte Effacement (LEE). Despite the most serious cases are generally associated with LEE-positive strains, a crescent number of cases has been related to LEE-negative strains, especially due to O113:H21 serotype that was already isolated from HUS outbreaks and sporadic cases. The alarming expansion of STEC infections in recent years is attributed to a combination of factors, including the modern practice to raising animals, changes in dietary habits in urban populations, the small infective doses required and the inability of conventional procedures and the inspection of food (sensory) to detect the pathogen. The soft white cheese is a product that has wide commercial acceptance in Brazil. Although the legislation required the use of pasteurized milk in its preparation, this legal regulations is not always met. Raw milk is an important vehicle of many intestinal pathogens such as *E. coli* pathotypes. The behavior of distinct STEC serotypes can vary in relation to physical and chemical properties found in foods and frequently used control measure procedures. So, the aim of this study was to evaluate the behavior of a O113:H21 STEC Brazilian strain (EC 784) isolated from food, under the individual and combined effect of four factors (pH, temperature, time and water activity - Aw) in soft white cheese. The effect of the four independent variables on the response (dependent variable) was assessed by using a central composite design experimental planning created by the software Statistica 7 (StatSoft, OK, USA). The assays were performed using as substrate the whey of soft white cheese with the variables set to mimic the conditions found in cheese. The experimental design involved the combination of the variables pH (5, 6 and 7), Aw (0.92, 0.94 and 0.96), time (0, 7 and 15 days) and temperature (8, 26 and 35°C), generating 27 runs, with a central point and two authentic replications of the experiment, amounting to 81 tests. From these experimental data the statistical program achieved the construction of an equation ($R^2 = 0.8$) which describes the variation of the microbial counts according to the variables. The results showed that the effect of all variables even individual or combined was significant over the response. Only at the condition of pH 7, Aw 0.94 and 26°C for 15 days there was an increase in the microbial population related the initial inoculum. In other assessed conditions, the number of viable cells has decreased. The condition of pH 5, Aw 0.96 and 35°C for 15 days, was shown to be the less favorable for STEC, since no viable cells recovering was possible in any of the repetitions. Despite their virulence potential previously described, the STEC strain studied (EC 784), seems not to have a distinct behavior in relation to other commensal *E. coli* strains.

Keywords: *Escherichia coli*, STEC O113:H21, soft cheese.

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**Betalactam resistance in food *Escherichia coli* isolated from broilers**

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*E. coli* is also a common inhabitant of the human and animal gut and is considered an indicator of fecal contamination in food. ESBL (extended spectrum beta-lactamases) producers are not very frequent in animals. TEM-1 is the most common variant among *E.coli*, however during last years TEM-52 ESBLs have been described in poultry and beef isolates. The aim of study was to compare minimal inhibitory concentrations (MIC) and betalactam resistance in *E. coli* strains isolated from poultry and turkey meat and faeces during six months period.

Eighty seven *E. coli* faecal isolates from various poultry and turkey farms showed relatively lower betalactam (ampicillin, ampicillin with sulbactam, ceftiofur, cefquinome) resistance in comparison with fifty one *E. coli* isolates from poultry and turkey meat bought directly in supermarkets. Similarly lower were levels of MICs to betalactams in faecal *E. coli*. High level betalactamases (TEM1,2/SHVI) were detected more frequently in *E. coli* isolates from meat (27%) compared to isolates from faeces (13,7%). ESBL TEM were determined by interpretative reading of MICs. ESBLs were present in 27% food strains while in faecal group *E.coli* were only in few strains. Integron 1 and virulence factors were detected by PCR in *E. coli* from both groups.

Results showed that source of surface meat microflora is not only faecal microflora, but also environmental microbes in poultry abattoirs. This is the first report of a quantitative antibiotic susceptibility testing and of phenotype resistance mechanisms determination in *E. coli* isolated from poultry meat in Slovakia.

This study was supported by slovak grants APVV-0028-07 and VEGA-0012-08.

Keywords: ESBL, *Escherichia coli*, food, environment
Biocontrol of the patulin-producing *Penicillium expansum* by yeast: *in vitro* and *in vivo* assays

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*Penicillium expansum* is one of the main fungal contaminants causing decay in fruits and is the main postharvest pathogen in apples. This fungus also produces patulin, a mycotoxin that causes acute toxic effects in humans and which maximum limits in foods for human consumption are under legal regulation in the EU.

Traditionally, *P. expansum* is controlled by the application of synthetic fungicides. Recently, biological control has been proposed as a feasible alternative. Yeasts possess many characteristics which are desirable in a biocontrol agent: not fastidious as to nutritional requirements, capable of growth in fermenters on inexpensive media and non-productive of secondary metabolites.

In this study we have analysed 16 yeast strains (of genus *Debaryomyces*, *Pichia*, *Saccharomyces*, *Zygosaccharomyces* and *Torulopsis*) in *in vitro* tests against 17 strains of *P. expansum* isolated from apples. The antagonistic effect was observed on YMA plates with methylene blue. *D. hansenii* and *P. anomala* showed the greatest antagonistic efficiency, particularly *D. hansenii* CYC 1244 strain, and therefore was selected for further *in vivo* studies.

In the *in vivo* studies, apples were wounded in three points with the sterile head of a needle and were subsequently inoculated with 20 µL yeast (1 x 10⁷ cell/mL), after two hours a 20 µL *P. expansum* suspension (1x10⁷ spores/mL) was added. The apples were stored at 4°C, 15°C and 25°C. A reduction of 30-50% in rot lesions was observed at 25°C, the most permissive temperature for *P. expansum*, and reached 75% at 4°C, a usual temperature for long term storage of apples. The main conclusion of the present study is that *D. hansenii* CYC 1244 is a good candidate its use in the integrated strategies to control *P. expansum* growth on apples and other fruits.

Keywords: Biocontrol, Debaryomyces hansenii, *Penicillium expansum*

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Characterization and identification of a bacteriocin produced by *Leuconostoc pseudomesenteroides* KM432BZ

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*Penicillium expansum* is one of the main fungal contaminants causing decay in fruits and is the main postharvest pathogen in apples. This fungus also produces patulin, a mycotoxin that causes acute toxic effects in humans and which maximum limits in foods for human consumption are under legal regulation in the EU.

Many food-grade microorganisms are used to produce a variety of fermented food. Lactic acid bacteria (LAB) are among the most important groups of microorganisms used in food fermentations. They contribute to the taste and texture of fermented products and inhibit food spoilage bacteria by producing large amounts of lactic acid and growth-inhibiting substances named bacteriocins [1]. Thus, research and development of probiotics is a field in full expansion.

Bacteriocins are antimicrobial peptides ribosomally synthesized by bacteria with an activity against related species. This has prompted new approaches to inhibit foodborne pathogens, in particular *Listeria* and *Enterococcus*. *Boza* is a fermented beverage from the Balkans prepared from cereals, such as rice, maize or wheat. Many LAB producing antimicrobial compounds have been isolated from this beverage [2, 3], such as *Leuconostoc* [4], which produces peptides active against *Listeria ivanovii*, a pathogen known to be responsible for animal and human infections [2, 5].

Here we isolated a bacteriocin-producing bacterium from *Boza* that we identified as *Leuconostoc pseudomesenteroides* by biochemical and molecular analysis. Purification of the bacteriocin was performed by ammonium sulphate precipitation followed by reversed-phase HPLC. Mass spectrometry analysis indicated a molecular mass of 3930 Da. The primary structure, determined by Edman degradation and ESI-MS/MS indicates that this antimicrobial peptide is a class Ia bacteriocin. Analysis of the genes involved in the bacteriocin biosynthesis need to be elucidated further.


Keywords: bacteriocin, lactic acid bacteria, food grade microorganism, boza, Leuconostoc pseudomesenteroides,
characterization and purification of natural food–biopreservative produced from Bacillus subtilis A12 isolated from a refreshing drink whey

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Whey is a popular refreshing fermented milk drink of North India. The whey consumption has been linked to the disease prevention and health improvement. In the present study, bacteriocin producing food grade bacterial strain isolated from whey has been identified as Bacillus subtilis by 16srRNA gene technique. Bacteriocin has been defined as extracellularly released primary or modified products of bacterial ribosomal synthesis which can have a relatively narrow inhibitory spectrum. Bacteriocin of Bacillus subtilis showed antagonism against deadly food borne pathogens viz, Listeria monocytogenes, Lactobacillus plantarum, Clostridium perfringenes. Bacteriocin of Bacillus subtilis was purified by single step gel exclusion chromatography and molecular weight was found to be 13 kDa by SDS-PAGE. Purified bacteriocin withstood temperature up to 121°C, found active at wider pH range and was sensitive to proteolytic enzymes viz trypsin and pepsin. The antibacterial substance showed bactericidal effect against sensitive indicators and it was stable for 3 months at 4°C.

characterization of Bacillus bacteriophage isolated from the fermented soybean, chungkookjang

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Recently, the fermented soybean products has drawn a great attention from people as a well being-healthy food. Chungkookjang, a traditional fermented soybean which is comparable with natto of Japan, has been consumed over many hundred years by Korean. Chungkookjang is made by fermenting the steam cooked-soybean with Bacillus subtilis for a couple of days. A high grade chungkookjang would be covered with copious amount of sticky substance (Fig. 1), which is identified as poly-γ-glutamate (γ-PGA), a metabolic product of Bacillus subtilis. Productivity of γ-PGA, the key substance of chungkookjang quality, is affected by the physiology of B. subtilis as well as bacteriophage infecting host strains.

To gain insights of bacillus phage in the starter strain of chungkookjang fermentation, a virulent bacteriophage (we named it as bacillus phage K2) isolated from chungkookjang products was investigated with respect to infectivity toward a number of B. subtilis starter strain, morphology, and its genomic nature including genome size and DNA restriction pattern.

Bacillus phage K2 showed a different lytic spectrum against numbers of B. subtilis strain. K2 is highly active against B. subtilis starter strains which were isolated from the Korean chungkookjang products, but much less active against a Japanese natto strain. Lytic activity of K2 phage toward host strains varied depending upon culturing temperature of host cell. For instance, a complete lysis of the K2 phage infected B. subtilis strains cultured at 37°C showed nearly complete lysis within 5-6 hours, whereas culturing at 42°C showed a poor lysis. Bacillus phage K2 is a tailed phage with an isometric icosahedral head (40 nm long of lateral side), a long contractile fiber (85-90 nm long, 15-20 nm wide), thin tail fibers (75-80 nm long, 6.3 nm in wide), and basal plate (12.6 nm long, 45 nm wide) with a number of spikes, but no collar. The shape of phage K2 differs from other bacillus phages such as SP01- like viruses or phi 29-like viruses in its size and detail structures. The bacillus phage K2 is rather a small DNA phage. When genome size was estimated by agarose gel electrophoresis and endonuclease analysis, it turned to be around 17 kb. Analysis of deoxyribonucleotide sequence of K2 genome and its gene structure is being undertaken for new insights on the bacillus phage that causes economic loss in chungkookjang industry.

Keywords: fermented soybean, chungkookjang, Bacillus subtilis, bacillus phage K2, isometric icosahedral tailed phage
Chitosan Matrices as Carriers for the Delivery of Natural Volatile Antimicrobials

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Chitosan [1] is a cationic linear amino polysaccharide obtained from partial N-deacetylation of chitin, one of the most abundant natural biopolymers present as a structural component of the exoskeletons of crustaceans, insects and other arthropods, as well as in the cell walls of certain fungi. Chitosan is biodegradable, biocompatible and non-toxic and displays specific properties due to the presence of free amino groups which make it a versatile polymer with a broad range of applications in several fields including biomedicine, pharmacy, agriculture and the food and paper industry.

β-Cyclodextrins are natural cyclic oligosaccharides consisting of seven α-1,4-linked units of D-glucopyranose and are obtained from the enzymatic degradation of starch. Cyclodextrin forms a toroidal truncated cone structure giving rise to an internal cavity with a relatively hydrophobic surface and an external hydrophilic surface. This shape allows the formation of inclusion compounds with a large variety of organic molecules.

The aim of this work has been to develop novel biobased matrices capable of carrying and releasing controlled amounts of biocidal compounds. For this purpose, chitosan in the form of chitosonium acetate was blended with hydroxypropyl-β-cyclodextrins (HP-β-CD) in the proportion of 1:1 (w/w) in aqueous media, and films were obtained by casting. The ability of the films to absorb carvacrol was evaluated after their immersion in the pure liquid.

The results showed that the incorporation of HP-β-CD in the polymer matrix greatly increased the sorption of carvacrol. However, the content of carvacrol in the composites was related to the initial concentration of glycerol and the degree of hydration of the polymer matrix. Thus, depending on the composition of the film, the chitosan matrix was able to hold up to 260% (g carvacrol/100 g dry film) of volatile compound. The amount of carvacrol retained in films lacking HP-β-CD and with different amounts of water and glycerol in their composition in no case surpasses 1%. Chitosan films exhibited long lasting release of carvacrol, exerting antimicrobial properties against E. coli and S. aureus after 20 days kept in air at 23 ºC and 40% RH. The new matrices could be used in the design of antimicrobial food preservation systems.

Keywords: chitosan, hydroxypropyl-β-cyclodextrins, carvacrol, sorption, antimicrobial films.

Comparison between Lactic Acid Bacteria populations present in two green table olives fermentative processes

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Introduction and Experimental Procedures. The main commercial table olives preparation in the international market is called “Spanish-style green olives”. Brines for this study were obtained from S.C.A. Olivarera Jesús Nazareno (Aguilar de la Frontera, Córdoba). The preparation is a fermentation process which can be carried out in different kinds of vessels [1]. Hence, buried vessels are used in the traditional elaboration system instead of fermentation warehouse. Both processes show significant differences as much structural as of handling, affecting mainly the temperature of fermentation and the final time of fermented olives. Without heating, the warehouse system maintains higher temperature than the buried vessel system and, therefore, it decreases the fermentation period [2]. In addition, the warehouse system with an installation of automated heating can maintain the temperature around 25 ºC, independently of the room temperature, which reduces significantly the processing time (in other words, it decreases the time needed to get the final table olives).

The aim of this study is to compare the Lactic Acid Bacteria (LAB) populations from the traditional elaboration system, which uses buried vessels, with those from the fermentation warehouse system. Samples of brine and temperature data were taken from the vessel at three heights (up, medium and down).

The brine solutions were used for microbiological analyses (pH, titratable acidity and salt content measurement). Colonies showing general characteristics of lactic acid bacteria were picked. Initially, Gram staining and catalase test were performed in order to identify the isolates. Lactic acid bacteria isolates were classified as L. plantarum and L. brevis mainly. The results showed that the incorporation of HP-β-CD in the polymer matrix greatly increased the sorption of carvacrol. Thus, there were important differences as far as the final time fermentations of the olives in both vessels, with the consequent repercussion of the price paid by the fruit. Likewise, total LAB populations were higher in fermentation warehouse.

References

Keywords: Olive fermentation, Lactobacillus, lactic acid bacteria.
Comparison of eleven *Escherichia coli* quantitative methods for Malaysian ready-to-eat food (poultry)

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Various international methods are available for *Escherichia coli* enumeration in food. These methods need to be validated with local food samples before they could be considered as national methods. Hence, this study was conducted in order to validate and compare eleven standard methods by using ISO 16140 procedure. The methods were pour, spread and drop plating, Petrifilm™, three types of direct plating and four types of MPN methods. Five types of ready-to-eat food (poultry) were spiked with three *E. coli* strains which were ATCC 25922, IMR 1/3 107B and IMR E243. The artificially contaminated foods were finally exposed to heat at 55°C for 4 to 6 min to stress *E. coli* before the eleven standard methods were applied onto the food. The correlations of data were analysed by using the Ordinary Least-Squares (OLS) Regression. All methods gave similarities for the recovery of stressed *E. coli* (p<0.05) in food (poultry). Practical approaches were also considered in order to identify the three best methods for the recovery of stressed *E. coli* in food (poultry). The observation found that pour plating, drop plating and Petrifilm™ were more practical than the other methods.

Keywords: Ready-to-eat food, OLS Regression, ISO 16140, *Escherichia coli*

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*Cronobacter sakazakii*, Enterobacteriaceae and microbial population of infant formula milk

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This study was carried out to detect and identify the presence of Enterobacteriaceae, *Cronobacter (Enterobacter) sakazakii* and microbial population of infant formulas in the Malaysian market. Samples that were analysed included 16 infant formulas (RB 1 - RB 16) and 14 special infant formulas (RB 17 - RB 30) from 8 manufacturers. The samples were obtained randomly from hypermarkets and private hospital in Ampang area. Aerobic plate count (APC), identification of Enterobacteriaceae and *C. sakazakii* and microbial population of infant formulas were carried out. A total of 5 samples (RB 11, 13, 18, 19 and 29) failed to comply with the microbiological criteria for APC as stated in the Malaysian Food Act 1983 and CAC. For the identification of Enterobacteriaceae, *Klebsiella pneumoniae* spp. pneumoniae, *E. cloacae*, *E. amnigenus*, *E. asburiae* and *Pantoea* spp. 3 were detected in 7 samples after confirmation using ID 32E biochemical test (Biomerieux). For the *C. sakazakii* identification test, EE broth and CSB were used as enrichment medium for the isolation of *C. sakazakii* on two types of chromogenic agar (CES and DFI) before the identity of presumptive colonies was confirmed with ID 32E. No *C. sakazakii* positive samples were detected after the confirmatory test. The combination of CSB and CES had the highest specificity (90.9%), followed by CSB and DFI (88.2%), EE and CES (81.1%), EE and DFI (78.9%) in the detection of typical *C. sakazakii* colonies (blue-green).

Keywords: Enterobacteriaceae, *Cronobacter (Enterobacter) sakazakii*, microbial population, infant formula
Dairy using kefir grains: production and development

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Kefir grains is originated from the Caucasus mountains in Russia, it is considered an initiator of natural lactic acid fermentation and composed of several bacteria and yeasts, in symbiosis. The maintenance of kefir grains can be performed into juice, molasses, sugar and any kind of milk, like cow, goat, sheep, camel, buffalo and soya milk; and the fermentation produce a single self-carbonated beverage and cool slightly, known as kefir. This study aims is develop the processing conditions to obtain a probiotic milk product using kefir grains, applying the surface response methodology. The response variables were viscosity, pH and the number of lactic acid bacteria into the product, it was used a randomized 2³, repeat the central point for evaluating the response variables according to the following variables: the concentration of milk powder (0, 6.25 and 12.5%), temperature (25, 35 and 45°C) and the concentration of kefir grains (5, 7.5 and 10%), in static fermentation. The variables were analyzed in the following range 0, 6, 18 and 24 hours. The best process condition obtained for the production of a product with high viscosity and high potential probiotic was 12.5% milk powder, 10% of kefir grains and a temperature of 45°C, which has viscosity of 2840 cP, pH value 3.53 and 1.83x10⁹ CFU/mL of lactic acid bacteria in 24 hours fermentation. According to Brazilian legislation, for a product to be probiotic is necessary that it contains the viable cells number in the order of 10⁶ CFU/mL, confirming that the product, is already considered a probiotic. The pH value at end of fermentation ranged from 3 to 6. Based on the results, it was conclude that the temperature rise and the addition of milk powder are significant variables (p< 0.05) to increase the viscosity, obtaining a milk drink with similar consistency marketed in the Northeast of Brazil and the temperature and concentration of grains of kefir significantly influenced (p< 0.05) for the higher potency of probiotic milk drink.

Keywords kefir, response surface, probiotic.

Damages to cattle dairy by intake of a corn based concentrate contaminated by Aspergillus flavus

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Aflatoxicosis is a public health problem in Brazil and in the world. It's one of the major sources of injury to the producers. Aflatoxins are produced by fungi of Flavi section, mainly Aspergillus flavus and A. parasiticus, Minas Gerais State, Brazil, is one of the biggest milk producers of the country. In 2008/2009 crop year, that was particularly rainy, it was observed a case where dairy cattle submitted to the intake of corn based concentrate showed the following symptoms: tapenade, ruminal atony, abortions in different stages of gestation and an acute fall on the milk production. Samples of the corn used in the concentrate were sent to the Microbiology Laboratory of EPAMIG in Lavras, Minas Gerais State. The grains were submitted to the Blotter Test and it was observed that 63% of them were infected by the fungus Aspergillus flavus, a potential producer of aflatoxins B1 and B2. This confirms that, beyond the injury to the cattle, there's the risk of exposure of humans, once the cows didn't die and their product (milk) was introduced into the food chain. Once milk is an important component of human diet, this work shows the importance of a severe inspection of the components of animal diets and when contamination is detected, the immediate condemnation of the lot. In these cases it isn't necessary the analysis of aflatoxins presence, once the state of deterioration of the grains can be clearly seen. So a detailed report about it, describing these aspects, signed by a professional of the area is already enough. It's relevant considering the high cost of these analysis.

Keywords corn, aflatoxins, dairy.
Detection and antibiotic susceptibility of coliform bacteria in fresh vegetables

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The consumption of fresh vegetables, which generally are eaten without further processing, is increasing as consumers strive to eat healthy food. These products carry natural non-pathogenic epiphytic microorganisms, although during growth, harvest and further handling, the produce can be contaminated with pathogens from human and animal sources. Contamination can arise as a consequence of treating soil with organic fertilisers, such as sewage sludge and manure, and from the irrigation water, as well as from the ability of pathogens to persist and proliferate in vegetables. Most of the reported outbreaks of gastrointestinal disease linked to the fresh produce have been associated with bacterial contamination, particularly with members of the Enterobacteriaceae family. In addition, the presence of antibiotic resistances both in normal flora and pathogenic microorganisms in fresh vegetables may contribute to horizontal spreading of resistances. Therefore, microbial contamination of fresh vegetables constitutes a relevant risk factor for consumer safety.

In this work we have determined the presence of coliform bacteria, as well as their antibiotic susceptibilities, in fresh vegetables as an indicator of their microbiological quality and their potential as a risk factor for consumers. We have studied ten samples from six different vegetables: two kinds of tomato, three of lettuce, and one of carrot. All fresh products were purchased from supermarkets and greengrocer’s shops in Valencia city (Spain). Coliforms were isolated in 50% out of the 60 samples analyzed, although only one isolate was identified as Escherichia coli. The identified species included enterobacteria: Klebsiella pneumoniae (n: 5), Klebsiella oxytoca (n: 10), Serratia marcescens (n: 1), Serratia rubidaea (n: 1), Enterobacter cloacae (n: 20), Kluyvera ascorbata (n: 2), and Pantoea agglomerans (n: 3), as well as other bacterial species: Acinetobacter baumannii (n: 1) and Stenotrophomonas maltophilia (n: 1). The susceptibility to eleven chemotherapeutic agents was determined in all bacterial isolates (n: 45). Most isolates were resistant to ampicillin (all except the S. maltophilia isolate and two P. agglomerans isolates) and most of them also to amoxicillin/clavulanic acid (all except the S. maltophilia isolate and the three P. agglomerans isolates). Resistances to other agents were sporadic: tetracycline (four resistant isolates), nitrofurantoin (four resistant isolates), sulphamethoxazole/trimethoprim (two resistant isolates), streptomycin (one resistant isolate), ceftoxime (one resistant isolate), and chloramphenicol (one resistant isolate). No resistances were found to gentamicin, ciprofloxacin, and cefadroxil. Only three bacterial isolates (two P. agglomerans and the one of S. maltophilia) were susceptible to all antibiotics tested, whereas three isolates (A. baumannii, E. cloacae and S. marcescens) showed multiresistance to four agents, and one isolate (E. cloacae) was resistant to five antibiotics. The presence of resistant bacteria in fresh vegetables arises mainly from the large amounts of antibiotics used in agriculture and animal husbandry, as applying manure from animal farming to agricultural fields or contamination of irrigation water can spread antibiotic resistant bacteria to plants.

The bacterial isolates from fresh vegetables include several opportunistic human pathogens, which may cause a variety of infectious diseases in the immunocompromised host and, in addition, antibiotic resistances can be horizontally disseminated, after ingestion by the consumer, to other gut commensal or pathogenic bacteria. Therefore, consumption of fresh vegetables may represent a potential risk factor for the consumer health, particularly in debilitated or immunocompromised individuals, and microbial contamination of these products can be considered as a food safety concern.

Keywords: coliform bacteria; fresh vegetables; antibiotic resistance

Detection and Characterization of Pathogenic Vibrios in Seafood by a PCR-LDR-Universal Array Approach

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Bacteria belonging to the genus Vibrio are Gram-negative motile rods widespread in the aquatic environments, particularly in the marine and estuarine waters (Hervio-Heath et al. 2002), on the surfaces of marine plants and animals (Baumann et al. 1984), and in the intestine of marine animals (Bergh et al. 1994). According to Bergey’s Manual of Systematic Bacteriology (2002), six genera and 46 Vibrio species are recognized within the current family Vibrionaceae. Several species are pathogenic for marine vertebrates and invertebrates, and some others are classified as human pathogens (Zhang and Austin, 2005). Though the vast majority of Vibrio is non-pathogenic for humans, 12 species are potentially harmful for human health. The Vibrio species of clinical significance include V. cholerae, V. vulnificus, and V. parahaemolyticus. Moreover, other vibrios e.g. V. alginolyticus, V. mimicus, and V. holilae – now taxonomically described as Grimontia holimae - have been sporadically found in infection outbreaks. Such events may be correlated to the horizontal gene transfer of virulence factors between members of the genus (Gonzalez-Escalona, 2009).

To assure seafood safety it is necessary to develop a rapid method for the specific identification of the harmful strains through the determination of their most relevant virulence factors. To this aim we developed a PCR-Ligase Detection Reaction-Universal Array (PCR-LDR-UA) approach for the identification of vibrios harmful strains occurring in the Adriatic Sea, through the simultaneous detection of species-specific markers and their most relevant virulence factors. A total of 11 genes on 6 Vibrio species were analyzed, through single or multiplex PCR.

A liquid phase LDR was performed using 28 species- and gene-specific probe pairs. The entire procedure was validated on 9 ATCC reference strains and 29 isolates from clinical and environmental samples, showing good specificity and sensitivity down to 6 fml of starting material. The possibility to perform multiplexed identification by PCR-LDR-UA platform was also assessed. The research also dealt with the identification of vibrios present in bivalves as they are filter feeders, often only a precooked or improperly processed. Direct DNA extraction and amplification was achieved from shellfish homogenate, overcoming the need of prior microbiological colony isolation. In order to verify the reliability of the detection, bivalve tissue homogenates were infected with different strains of target Vibrio species, trying to mimic the complexity of the matrices commonly analyzed by standard microbiological procedures. Samples were processed for DNA extraction and amplification and then analyzed by the LDR-UA procedure. The results confirmed the detection power and specificity of the procedure as all the target species used for the infection were identified and all the expected marker genes were correctly detected. The reliability of the assay was further confirmed by the screening of 13 environmental shellfish samples destined to human consumption (Tapes philippinarum, Mytilus galloprovincialis, Crassostrea gigas). The LDR-UA assays confirmed the presence of vibrio as indicated by the PCR screening, but not detected by the traditional biochemical screening.

Our approach was demonstrated as a reliable and sensitive detection method for identification of harmful Vibrio strains in seafood. To this purpose the LDR-UA approach was applied and, as far as it is known, it was the first application of this technique for pathogen detection in seafood.

Keywords: Pathogenic Vibrio, Seafood, LDR, Universal Array, virulence factors.
Detection of Lactobacilli from Fecal Flora of Some Infants

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During the past two decades identification of lactobacilli isolated from normal flora has received great interest due to their health promoting effects. This study has aimed at characterizing the lactobacilli strains isolated from the fecal flora of Iranian infants based on phenotypic oriented methods. Moreover, the diversity of identified species among tested infants has been looked into. Thirty two strains of lactobacilli were included in this study. The given strains were previously isolated from the fecal samples of 6 infants between 1-19 months of age. They were examined through 14 carbohydrate fermentation tests, growth ability at different temperatures and in the presence of different concentrations of NaCl. Cell and colony morphology were assessed as well. The examined strains were identified as L. acidophilus (12 strains), L. plantarum (9 strains), L. rhamnosus (7 strains), L. paracasei (3 strains) and L. fermentum (1 strain); 2 strains remained unidentifed. Accordingly L. acidophilus was the most predominant species among the tested samples. Some biochemical differences were observed among the strains of L. acidophilus group and some morphological peculiarities were obtained among the strains of L. paracasei and L. rhamnosus in comparison to the typical strains of L. casei group. These differences revealed the necessity of application of complementary molecular methods for clear identification of examined Lactobacillus strains.

Keywords: Lactobacilli, fecal flora, phenotypic techniques.
Discrimination of bacteria using optic fiber-based in situ synchronous fluorescence spectroscopy of colonies

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In the field of medical, environmental or food analysis spectroscopic methods are gaining increasing interest. Among them, the Raman and Fourier transform (FT)-infrared spectroscopies are the most finalized for the bacterial discrimination purpose. But fluorescence methods offers several inherent advantages compared to the FT-infrared for example: it is 100 to 1000 times more sensitive allowing to investigate a given molecule; while another major interest of fluorescence is the absence of signal from water. Bacteria contain several intrinsic fluorophores that emit photon following excitation in the ultraviolet region. Trytophan, phenylalanine and tyrosine are some of the most common fluorescent molecules. The nucleotides could also fluoresce, but their quantum yields are about 100-times lower than the quantum yields of tryptophan. In addition, several enzymes or cofactors, such as reduced nicotinamide adenine dinucleotide (NADH) and riboflavin being the most prominent examples, exhibit pronounced native fluorescence after excitation set at 320 nm and 380 nm, respectively. In the case of colonies on agar plates, bacterial populations are highly heterogeneous in terms of physiologic state, and genotypic and phenotypic status and are embedded in complex polysaccharidic matrices. It is probable that numerous intrinsic fluorophores co-occur, with overlapping spectra, causing a loss of information when analyzed at one set excitation wavelength. That’s why we explored the potentialities of synchronous scanning fluorescence spectroscopy (SyF). According to this technique, both the excitation and emissions monochromators are scanned simultaneously in such a manner that a constant wavelength interval is kept between emission and excitation wavelength (Δλ). Using suitable Δλ and step analysis, SyF reduces spectral overlapping by narrowing spectral bands and simplifying spectra. This technique is very useful for the study of mixtures of fluorescent compounds. It is largely used in the fields of oil, pharmaceutical, and specific aromatic hydrocarbon analysis but has never been used for bacterial analysis to our knowledge.

This work presents the application of the method for Pseudomonads characterization, which form one of the most adaptable flora and present the biggest phenotypic and genotypic diversity so far depicted. The reference species were selected in order to cover representatives of phylogenetically unrelated taxa (Burkholderia, Xanthomonas, Burkholderia and Stenotrophomonas), and, within the genus Pseudomonas, relatively unrelated (P. stutzeri versus P. chlororaphis) as well as related species e.g. P. chlororaphis, P. fragi, P. lundensis and P. taetrolens. Several biotypes of a same species were also tested (P. putida: 3 strains, P. fluorescens: 4 strains, P. fragi: 2 strains, P. chlororaphis: 2 strains, P. stutzeri: 2 strains, P. syringae: 2 strains).

Synchronous fluorescence spectroscopy was successfully developed coupled with acquisition of spectra directly from colonies on agar plates using an optic fiber. The variance analysis has shown an excellent repeatability of the results, but also no significant effects of “the optical fibre position in the colony” nor of the incubation time before reading (between 48 and 72 hours). Sensitivity and selectivity reached 100% for bacterial discrimination at the genus, species or subspecies level.

The method appears as a very reliable tool for a taxonomic purpose since our results are in agreement with the generally admitted rRNA and DNA bacterial homology grouping but they also bring out additional information about strain relatedness.

**Keywords:** Fluorescence spectroscopy, synchronous, Pseudomonades, Optical fibre, chemometrics, bacterial traceability.

Effect in *vitro* of lactic acid bacteria isolated from *guirra* sheep against *Salmonella* spp

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*Salmonella* is food borne pathogen associated with processed poultry and may cause severe illness as inflammation in ileum and colon and even death in human. *S. enteritidis* can grow substantially in fermented milks because they have the ability to adapt and proliferate between pH 2 and 4. It invades enterocyte-like Caco-2 cells, Peyer’s patches and M cells of the small intestine are the first to be invaded. There are experiences about the role of lactic acid bacteria in the inhibition of growth, prevention and treatment of gastrointestinal disorders for *S. typhimurium* and *S. enteritidis.*

Lactic acid bacteria is present in human intestinal tract and in different environments as milk, meat, vegetables, fermented products, etc. “Guirra” sheep is an animal indigenous from Valencia, in Spain. Guirra’s milk seems to contain Lactic Acid Bacteria (LAB) with probiotic potential.

**Objective:** To determine *“in vitro”, antimicrobial effects of lactic acid bacteria isolated from “guirra” sheep milk on* *S. typhimurium* and *S. enteritidis*. strains.

**Methods:** 131 strains isolated of sheep, were identified by CH50 API system. Four strains of *Salmonella* genus of different sources (reference, clinical, food and faeces) were used for the antimicrobial assays. Inhibitory activity was assayed both, with agar discs containing lactobacilli plated on a *Salmonella* culture plates and with “well test” method by adding 50 μl of the lactic acid bacteria MRS broth culture, without cells, in wells performed on Salmonella plates.

**Result:** Lactic acid bacteria strains isolated from Guirra’s milk were identified as L. acidophilus, L. brevis, L. delbrueckii, L. paracasei paracasei, L. plantarum, L. rhamnosus, Lc. lactis, Lc. raffinolactis, Pediococcus pentosaceus and Leuconostoc. Nineteen out of 131 isolated strains showed inhibitory activity. The antimicrobial capacity of each isolate depends on the method used and the Salmonella strain tested. Antibiotic resistant *Salmonella* strains were also less sensitive to lactic acid bacteria inhibitory effect.

**Keywords:** Lactic acid bacteria, *S. typhimurium*, *S. enteritidis*, antimicrobial activity.
Effect of methyl-2-benzimidazol carbamate and physicochemical factors on the growth and ochratoxin A production by *Aspergillus ochraceus* in bee pollen medium

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Bee pollen is a natural, highly appreciated food product due to its high content of proteins and free amino acids. It also contains carbohydrates, lipids, including fatty acids and sterols, vitamins and minerals. It is usually included in the diet of highly exigent consumers.

The natural mycobiota occurring in bee pollen and production of mycotoxins in this substrate was previously studied. It has been reported that some fungi produce influences either positively or negatively the production of mycotoxins. The aim of this study was to examine the efficacy of the fungicide methyl-2-benzimidazol carbamate (carbendazim) to control mycelial extension of an ochratoxigenic strain of *Aspergillus ochraceus* and its effect on ochratoxin A (OTA) production by that strain in bee pollen medium. The effect of different fungicide doses (0.01 – 5 mg/l) at water activities (a_w) ranging from 0.99 to 0.94 was studied.

The strain of *A. ochraceus* was deposited at the collection of the Department of Microbiology and Ecology, University of Valencia (Spain) (ref. Aso2) and at the Spanish Collection of Type Cultures (CECT, University of Valencia, Spain) (ref. CECT 20510), where its identity was confirmed. A solid medium containing 2% bee pollen, water and agar was used in this study. Cultures were maintained at 25°C for two weeks and mycelia extension rates were measured over time. Lag phase for growth was considered as the time to reach a colony 5 mm diameter. For each a_w-value and fungicide dose, five Petri dishes were prepared and two right-angled diameters of the colonies were randomly chosen and measured every day until the colony filled the whole dish or the cultures were analysed for OTA by liquid chromatography with fluorescence detection.

Lag phases lasted two days at fungicide doses ≤ 2 mg/l regardless of the a_w-value. The highest lag-times (6 – 7 days) were observed at 5 mg/l and 0.98 a_w. They decreased to 3 – 4 days at 5 mg/l and 0.96 a_w. No growth was observed in cultures at 5 mg/l and 0.99 a_w.

The growth rate of *A. ochraceus* decreased at fungicide concentration > 2 mg/l. The highest growth rate was observed at 0.98 a_w. OTA production in cultures was studied as a function of a_w and fungicide concentration. The highest OTA level was found at 0.05 mg/l and 0.99 a_w. Addition of carbendazim at the levels assayed inhibited OTA production in cultures at 0.96 and 0.94 a_w. However, at 0.99 and 0.98 a_w, OTA was detected only at carbendazim doses ≤ 2 mg/l.

**Keywords:** Ochratoxin A; carbendazim; *Aspergillus ochraceus*

**Acknowledgements:** The authors wish to thank financial support from FEDER and Spanish Government “Ministerio de Ciencia e Innovación” (Project AGL2007-66416-C05-01/ALI, and two research grants).

Effect of pure and mixed cultures of the main wine yeast species on grapemust fermentations

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The non-*Saccharomyces* species were considered to be of secondary significance or undesirable to the wine process. However, this trend is changing. In a recent review, Fleet (2008) discussed the possibilities of using yeast other than those in the genus of *Saccharomyces* for future wine fermentations and the commercial viability of mixed cultures. These species present a great potential to introduce appealing characteristics into wine improving its organoleptic quality. The major non-*Saccharomyces* yeasts present during alcoholic fermentation is *Candida zemplinina* and *Hanseniaspora uvarum*. Although a clear reduction in the population size of these species happened throughout wine fermentations, several quantitative ecological studies indicated that its growth was not completely suppressed, either in spontaneous or in inoculated fermentations (Hierro et al., 2006; Hierro et al., 2007; Andorra et al., 2008). Consequently, the impact of non-*Saccharomyces* yeasts on wine fermentation cannot be ignored. They introduce into the process an element of ecological diversity that goes beyond *Saccharomyces* species and they require specific research and understanding to prevent any unwanted consequences they might cause or to exploit their beneficial contributions (Fleet, 2008).

Thus, mixed inoculation between non-*Saccharomyces* and *S. cerevisiae* yeasts are of interest for the wine industry for technological and sensory reasons. We have analyzed the effect of mixed inoculums of these main non-*Saccharomyces* yeasts and *S. cerevisiae* upon the fermentation performance, on the amino acid consumption, volatile compound and acid organic production in Macabeo fermentation, as well as the interactions among the different microorganisms involved. Sterile must was fermented, this were performed in triplicates in six conditions: 3 pure cultures of *S. cerevisiae*, *H. uvarum* and *C. zemplinina*, and the mixtures of *H. uvarum*: *S. cerevisiae* (90% and 10%) and *H. uvarum*: *C. zemplinina*: *S. cerevisiae* (45%, 45%, and 10%).

In the fermentations studied, the presence of non-*Saccharomyces* yeasts slowed down the fermentations, *H. uvarum* was the species which presented the slowest rate of fermentation and only pure *H. uvarum* fermentations were unable to finish the fermentation. We have observed the differences in the growth of our selected strain of *C. zemplinina* being able to finish the alcoholic fermentation in a reasonable time and in the mixed fermentations, the proportion between *S. cerevisiae* and the non-*Saccharomyces* yeasts being preserved until mid fermentation, when the levels of *S. cerevisiae* were in the 10^2 cells/ml range. The only difference with the quick imposition of the mixed inoculum of the selected strain of *C. zemplinina* for OTA by liquid chromatography with fluorescence detection.

In the fermentations studied, the presence of non-*Saccharomyces* yeasts slowed down the fermentations, *H. uvarum* was the species which presented the slowest rate of fermentation and only pure *H. uvarum* fermentations were unable to finish the fermentation. We have observed the differences in the growth of our selected strain of *C. zemplinina* being able to finish the alcoholic fermentation in a reasonable time and in the mixed fermentations, the proportion between *S. cerevisiae* and the non-*Saccharomyces* yeasts being preserved until mid fermentation, when the levels of *S. cerevisiae* were in the 10^2 cells/ml range. The only difference with the quick imposition of the *S. cerevisiae* in industrial wine making was the inoculated population, which was 10 times lower than the regular inoculation practice. Furthermore, the ethanol production do not seem to have a great influence by the yeast involved on fermentation, with the exception of *Hanseniaspora* which can not finish the sugar content. The non-*Saccharomyces* yeast produced higher levels of glycerol and acetic acid. Mixed fermentations produced higher consumption of available amino acids as well as higher complexity in the synthesis of volatile compounds. However, the amount of acetic acid well above the admissible levels compromises an immediate application of mixed cultures.

**Keywords:** Saccharomyces, Candida, Hanseniaspora, wine fermentation, volatile compounds, amino acids.

**References:**


**Keywords:** Ochratoxin A; bee pollen, *Aspergillus ochraceus*
Effect of the inoculum characteristics on the first stages of growing yeast population in beer fermentations by means of an Individual-based Model

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The budding yeast Saccharomyces cerevisiae has a limited replicative lifespan. The cell mass at division is unevenly partitioned between a bigger, old parent cell and a smaller, new daughter (virgin) cell. Industrial fermentation performed to produce beer is the unique within the alcoholic beverage industry in that the yeast is maintained and reused a number of times. At the end of fermentation a portion of the yeast is ‘cropped’ from the fermentation vessel for ‘serial repitching’. Typically this is the centre-top portion of the yeast crop, commercially comprising middle-aged and virgin cells. However, increasingly yeast is removed early to decrease process time via a ‘warm’ or ‘early’ cropping regime and this facilitates removal of the lower portion of the crop, comprising a greater proportion of aged cells. Harvesting yeast may therefore select a population with an imbalance of young and aged individuals. In fact, the output of a bioprocess is strictly dependent on the physiology of each single cell in the population, on the distribution of the cells throughout the cell cycle and on the effects of environmental conditions on the population. Unlike continuous models, Individual-based Modelling (IBM) is a bottom-up approach, meaning that it considers each microbe as an individual, a unique and discrete entity, with more than one characteristic that changes throughout its life. IBMs are in an increasingly established approach to diverse microbial communities and their use is also becoming more widespread in food microbiology. Of those available we have used INDISIM, the simulator developed by our group, and which has already been used to study different features of bacterial growth, providing an ample pool of interesting results [1]. INDISIM-YEAST constitutes the adaptation of INDISIM to study the specific characteristics of the yeast cell cycle and to deal with yeast populations growing in liquid media [2]. The aim of this contribution is, by means of individual-based simulations of INDISIM-YEAST, to explore the effects of inoculum characteristics of the yeast cell cycle and to deal with yeast populations growing in liquid media. [2] Ginovart M. and Cañadas J.C.: INDISIM-YEAST: an individual-based simulator on a website. Journal of Industrial Microbiology and Biotechnology, 35 (2008), 1359-1366.

Acknowledgements: This study was supported by funds from the Andalusia Government (EXP 92162/11) and the project RM2007-00508-C02-00 from INIA (Ministerio de Educación y Ciencia de España).
Effects of oxidative stress on viability and selected characteristics of probiotic bacteria

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Abstract
Probiotic bacteria as an alternative to antibiotics are gaining popularity worldwide (Mattila-Sandholm et al., 2002). Large amount of probiotic bacteria has been supplemented with dairy products and nutraceuticals and the biggest hurdle for maintenance of bacterial viability in oxidative damage as a result of exposure to high oxygen environment. The aim of this research is to screen probiotic bacteria for oxidative stress on the basis of physiological, biochemical and cellular characteristics and to identify the key physiological traits responsible for their survival at oxygen-rich atmosphere.

The RBGR (Relative bacterial growth ratio) study was performed for the selection of four strains (oxygen sensitive and resistant strains) from total of eleven probiotic bacterial strains. Confocal laser scanning microscopy (CLSM) was used for the determination of variation in concentration of viable bacterial cells, using Bac-light LIVE/DEAD viability kit, the results indicated that, 38% of Lc1 (O2 sensitive), 12% of DR20 (O2 resistant), B. lactis Bb12 (O2 resistant) strain observed 52% and 18% of B1912 (O2 resistant) cell growth was reduced when comparing the treated (21% O2) to the control (0% O2) bacteria.

The survivability in aerobic conditions is considered to be a significant factor that influences physiological activities of probiotic bacteria. Up to date there is limited research that utilizing proteomics to observe protein expression changes due to the oxidative stress from probiotic bacteria. From proteomic study it has been found that, the three probiotic bacterial strains showed varying results in protein expression because of oxygen treatments on the cells. Both Lc1 and DR20 expressed three proteins by 4-fold or more and 118 proteins by 2-fold or more were either up regulated or down regulated. Three proteins that were differentially expressed by 3-fold or more were identified by MALDI-TOF MS/MS and the MASCOT and BLAST databases. Also, 52 proteins were up regulated by 4-fold or more and five proteins were down regulated by 4-fold or more when comparing the control (21% O2) to the treated (0% O2) B. infantis strain. Nine and twelve up regulated and down regulated proteins, respectively, were subsequently identified by MALDI-TOF MS and the MASCOT and BLAST.

In addition, probiotic characteristics such as, acid and bile tolerance, hydrophobicity assay and cell adhesion capacity was measured to evaluate the physiological basis of oxidative tolerance. This data may more helpful for the adaptation of oxidative stress resistant in probiotic bacteria and potentially useful to improve viability in fermented dairy products.

Keywords: Probiotic bacteria, Oxidative stress, Physiological.

References

Evaluation of the activity of different fungicides against ochratoxigenic Aspergillus spp. in barley-based medium

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Barley is a nutritious cereal that contains carbohydrates, protein, vitamins and minerals among other nutrients. It is the fourth largest cultivated cereal crop in the world. In 2007/2008 crop the world total production of barley was 133 millions metric tons. Fungal species can grow in barley and produce great losses in crops. Some of them can produce mycotoxins that contaminate grain and can be hazardous to consumers. Two Aspergillus sections are known to produce ochratoxin A (OTA). They are the section Circumdati (the Aspergillus ochraceus group) and the section Nigris (Aspergillus carbonarius and Aspergillus niger aggregate). In Spain, A. carbonarius and A. ochraceus are the most frequently isolated ochratoxigenic species in barley. Application of chemical fungicides is the most widely used strategy to control fungal infection in cereal crops.

A study on the application of three fungicides (mancozeb, copper oxychloride and sulfur) on the growth of ochratoxigenic strains of A. carbonarius and A. ochraceus was performed in barley-based medium cultures. Water activity was 0.97 and temperatures were 15 or 25°C. Lag phases and growth rates were determined for each fungicide in these conditions. A statistical treatment of the data was carried out.

Mancozeb inhibited fungal growth at 30 mg/l while at 10-20 mg/l and 15°C it provided lag phases > 24 days. Copper oxychloride at 15°C proved inhibitory at 500 mg/l but at 25°C it did not delay fungal growth with respect to controls without fungicide. Sulfur proved inhibitory or provided large lag phases at 3-8 g/l at 15°C. However, at 25°C and up to a dose of 8 g/l growth of the strains was unaffected. The three fungicides were more effective (i.e. produced shorter lag phases and higher growth rates) at 25°C than at 15°C. Mancozeb was more effective than copper oxychloride, which was more effective than sulfur. The response of both isolates to each chemical was similar or different depending on the dose.

The efficacy of these fungicides on the growth of both species of Aspergillus in barley-based medium has not been reported previously.

Keywords: barley-based medium; fungicides; Aspergillus spp

Acknowledgements: the authors wish to thank financial support from FEDER and Spanish Government “Ministerio de Ciencia e Innovación” (Project AGL2007-66416-C05-01/ALI, and two research grants).
Evaluation of the persistence of viable *Listeria monocytogenes* cells in chlorinated water

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**Background.** *Listeria monocytogenes* is the causal agent of one of the most important foodborne diseases worldwide. *Listeria monocytogenes* is widely distributed in the natural environment. Being tolerant to adverse conditions such as extreme pH, high temperature or nutrient starvation, it can be found in soils, water, effluent and foods (Liu, 2006). The extended distribution of *L. monocytogenes* in the environment and its ability to persist in food-processing environments cause the frequent contamination of foods, which represents the main source of human infection. Our objective was to assess the effect of chlorine water treatment on *L. monocytogenes* and to study this organism’s survival strategies in chlorinated water.

**Materials and Methods.** *Listeria monocytogenes* NCTC 930 was inoculated into chlorinated water with different concentrations of free chlorine (0.07, 0.16 and 1 mg/L). Samples were aseptically removed after 10s, 1 min, 5 min, 2h, 16h and 24h. RNA content, 16S rRNA (FISH), DNA content (16S rDNA and hlyA gene), cultivability and substrate responsiveness combined with FISH detection (DVC-FISH assay) were assessed.

**Results.** *L. monocytogenes* cell cultivability was lost at 2h in drinking water with 0.16 and 1 mg/L of free chlorine. Number of *L. monocytogenes* cells with membrane damage was increased after 1 min in contact with 1 mg/L chlorinated water, but viable cells were detected until 2 hours. Viability was conserved for more than 16 hours at minor chlorine concentrations. Both, the 16S rDNA gene amplicon and the hlyA fragment specific for *L. monocytogenes* were detected after a 24-hour chlorine exposure. The analyzed bands intensity was constant throughout. 16S RNA levels were constant during chlorine treatment, thus chlorine-killed bacteria are unlikely to involve ribosome degradation.

Combining modified DVC and FISH techniques can rapidly and specifically detect and identify viable *L. monocytogenes* cells in water samples. Some normal disinfection practices used in drinking water treatment (free chlorine lower than 0.2 mg/L) proved to be inadequate to control this organism what could pose a public health risk.

Fluorescence *in situ* hybridisation for direct quantification of Saccharomyces cerevisiae and Hanseniaspora guilliermondii populations during alcoholic fermentations

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Both morphophysiological and molecular methods have been used to monitor the growth population dynamic of the indigenous yeast populations during wine fermentations. While classical methodologies are laborious, time-consuming and somewhat unreliable, genotyping methods, such as DGGE, RFLP, RAPO, MSP-PCR, electrophoretic karyotyping and optimized interdelta sequence analysis, have been successfully applied to differentiate yeast species and strains.

Using plate-depending methods, spontaneous wine fermentations show a typical yeast growth pattern where the less fermentative non-*Saccharomyces* species grow during the early stages, being the final stages invariably dominated by the alcohol-tolerant *S. cerevisiae* strains. However, the recent use of direct molecular methods (e.g. DGGE, qPCR) have allowed detecting yeast species throughout the fermentation process that were previously unnoticed by plate-depending methods (Andorrà et al. 2008).

Fluorescence *in situ* hybridisation (FISH) is a molecular method that uses fluorescein-labelled DNA oligonucleotide probes targeted to the complementary sequence of the ribosomal RNA. This technique combines the direct visualisation of the cells with the reliability of molecular methods. In wine-related applications, it has been used both for the rapid monitoring of lactic acid bacteria (Blasco et al., 2003) and for the detection of the slow growing yeast *Dekkera bruxellensis* (Stender et al., 2001). In a recent study, Xufre et al. (2006) applied this method, in combination with plate counts, to follow the evolution of yeast populations in two winery fermentations of white and red grape musts. In both cases, a high diversity of non-*Saccharomyces* yeast species was detected, including *Candida stellata*, *Hanseniaspora uvarum*, *H. guilliermondii*, *Klyveromyces marxianus*, *K. thermotolerans* and *Torulaspora delbrueckii*.

In the present work, FISH probes, specifically designed for *S. cerevisiae* and *H. guilliermondii* and labeled with FITC, were used to quantify the cell density of each yeast species during the course of single and mixed fermentations. FISH methodology was applied to daily samples and hybridised cells enumerated on polycarbonate filters using an epifluorescent microscope. Cells were doubled stained with DAPI and with FITC, which allowed quantifying the efficiency of the FISH hybridization. Culture-independent cell density profiles obtained from both DAPI stained-cells counts and FISH-probes hybridized cells were also compared with viable cells profiles obtained from the classical plate-counting method.

**Keywords:** fluorescence *in situ* hybridisation; direct monitoring of yeast population evolution; wine fermentations;

**References:**


Fungi associated with coffee berries in different ripening stages and submitted to five bagging times

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Coffee berries were harvested and separated according to different ripening stages (green/cane green, cherry, overripe/dry and fruit mix), then stored in braided polyethylene bags and submitted to five bagging times which varied between 0, 1, 2, 3 and 4 days. As each bagging time ended, we collected samples and submitted them to microbiological analysis in order to determine the dominant fungi genera associated with the berries and to observe the behavior of these microorganisms in the fermentation processes. The results demonstrated that the bagging process caused variations in diversity and in intensity of fungal microbiota for each ripening stage, and also that a longer bagging period favored the development of a yeast in the cherry stage that inhibited filamentous fungi growth. The overripe/dry stage demonstrated a greater occurrence of fungi section Circumdati and Nigri. All isolates of section Circumdati were identified as Aspergillus ochraceus Wilhelm, which are producers of ochratoxin A, and the amount of sclerotia varied according to the intensity of fluorescence. When the toxigenic potential of the isolates was tested by the Plug Agar technique, Aspergillus niger var niger and Aspergillus flavus failed to produce fluorescence. In the in vitro test the yeast did not affect the development of isolates of Aspergillus niger var niger, yet it reduced mycelial growth of Aspergillus ochraceus.

Keywords: coffee, fungal microbiota, fermentation, ochratoxin A.

Fusarium spp. occurrence in feeds and cereal grains (Portugal-2007)

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Fusarium spp. is an extended genus of moulds belonging to Ascomyceta group. Some of these moulds play a negative role on plant health and in the preservation of nutritional value of crops and feeds during its primary production and commercial life. Its growth in those matrices allows to nutrients losses and may enhance to toxicological effects. Fusarium spp. are moulds refered as one of the most problematic on feed safety. Growth of some Fusarium spp. strains in crops enhance a huge range of mycotoxins, with a special reference to trichothecenes, zearalenone, fumonisins, moniliformin, Toxic T2 and H-T2 toxin. These natural toxicants pose a threat to human and animal health; even for plant health they have also been found hazardous. The aim of this paper is to highlight the hygienic and sanitary meaning of the occurrence of Fusarium spp. in cereal grains used for animal nutrition (corn, oats and barley) and compounded animal feeds (poultry, swine and bovine) traded in Portugal. Fusarium spp. were searched and enumerated in 295 samples of feed and cereal grains during 2007. Samples were analysed using an internal official mycological method, performed in a duplicated procedure. Poultry feed (N=50) showed Fusarium contamination in 15 sample (30.0 %). Samples of swine feed (N=75) revealed the presence of Fusarium in 45 samples (60.0 %). Bovine feed (N=35) had those mould genera in 12 samples (34.3 %). In 37 samples of corn, Fusarium were detected in 32 (86.5 %). Oats (N= 45) revealed to be contaminated with Fusarium in 20 samples (44.4 %) and in barley (N= 53) those moulds were also frequent (n=39, 73.6%). The general level of registered contamination ranged from 1.5 log10 cfu/g to 5.3log10 cfu/g. Higher levels of contamination with Fusarium spp. were found in oats and corn. No positive sample of feed revealed contamination above 2.5log10 cfu/g. The small number of positive granulated feeds showed a very low level of contamination with Fusarium spp. Contamination levels of feeds are affected by the percentage of incorporation of each cereal and by the technological procedure: hot treatments (granulation). Although the low number of mould cells present in feed do not excluded the possibility of the presence of its toxic metabolites in feed, because these biological toxicants are chemical compounds are more resistant to thermal treatments than fungi cells.

Keywords: Fusarium spp; feed; contamination
Genetic diversity of *Streptococcus thermophilus* strains isolated from plant sources


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To date, the search for starter cultures has relied on surveying a large number of isolates, preferably selected from natural sources as the use of limited starters over the years limited their biodiversity. So in order to add new strains, study their heterogeneity and establish their habitat, 72 isolates of *S. thermophilus* have been isolated from vegetables, fodder crops and fermented cereals from 15 different regions of India. The isolates displayed the major phenotypic characters of *S. thermophilus* and also genotypically confirmed by species specific PCR using *lacZ* gene.

Thirty isolates were selected each representing different regions and kind of sample which included twenty five isolates ... 16S rDNA gene of all 30 isolates was amplified using group specific primers 2F S-D-Bact-0011-a-S-17 and 2R S-G-Lab-0677-a-A-17. Except 6 isolates viz., UUtCt (82%), UKSp2 (88%), UKFM1 (85%), UKD3 (87%), UKD2 (98%), UKCF (95%), the partial sequencing of the 16S rDNA of remaining isolates demonstrated high similarity (99-100%) with 16S rDNA sequence of *S. thermophilus* available in the NCBI GenBank database (FJ172679, FJ172680, FJ667758-FJ667772, FJ982785-FJ982797). Phylogenetic analysis of 16srDNA sequences resulted in 6 major clusters by UPGMA using Mega software (Version 4.1). The genetic distance of UUtCt and UKFM1 is found to be more than the other genotypes. Majority of the isolates were found to be moderate acidifiers i.e., capable of producing 0.5 to 0.6% lactic acid (LA) after 6h and fast acidifying activity (>0.6% LA) was found in only 2 isolates, UKFe and UKCu1. Among 11 flavor exhibiting isolates, 10 were found to be plant isolates and 21 were capable of producing capsaicin poly saccharide. In contrast to commercial strains, 9 were known to be urose negative and 5 isolates were galactose fermenters. Only plant isolates viz., UKsp1, UMbc, UIc1, UIHP, and UIpR were known to utilize galactose sugar which is a desirable technological trait. Majority of the isolates exhibited weak proteolytic activity ranging from 1.58 to 16.03 μg of leucine mL⁻¹ which is in accordance with *S. thermophilus* reference strains. Yogurt prepared using *S. thermophilus* plant isolates and standard Lb. delbruckeii ssp. bulgaricus (NCDC 09) was evaluated for sensory attributes received acceptable scores equivalent to that of reference dairy cultures. Hence, plant sources can also be a source of interesting new strains with specific functional properties, for their potential use in development of new starter cultures. Optimal exploitation of them requires further more detailed studies of their technological properties and performance in small-scale food fermentations.

**Keywords:** Isolates from plant sources; *Streptococcus thermophilus* characterization; 16sDNA partial sequencing; technological properties; yogurt starter

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Glucosidases of *Lactobacillus brevis* and *Oenococcus oeni* for aroma release in wine

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Lactic acid bacteria (LAB) are responsible for olfactory changes in wine during malolactic fermentation (MLF). A side characteristic of MLF is the release of grape derived aroma compounds from their glycosylated precursors by β-glucosidase activities of these bacteria. Therefore, several LAB strains were isolated from wine and malolactic starter cultures. Screening for glucosidase active cells was performed on plates with 4-methylumbelliferyl-β-D-glucopyranoside (β-MUG) and 5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside. Microscopic identification, Gram staining, catalase tests and restriction analysis of the amplified 16s rDNA gene were performed. The identified *O. oeni*, *L. hilgardii* and *L. brevis* strains were glucosidase positive. The *L. brevis* strains showed β-glucosidase, β-xylodase and α-arabinosidase but not α-rhamnidosidase activity.

The β-glucosidase of a *L. brevis* strain with high intracellular activity was purified and characterized. The pure enzyme is a homotetramer of 330 kDa. The Km values for p-nitrophenyl-β-D-glucopyranoside and p-nitrophenyl-β-D-xylpyranoside were 0.22 mM and 0.14 mM, respectively. The β-glucosidase is partly inhibited by glucose but not by fructose. Ethanol (12.5%) increases the activity up to 200%. The protein sequence was identified by LC-ESI-QTOF-MS/MS analysis of its tryptic peptides. A similar sequence was identified in the genome of *Oenococcus oeni*. The β-glucosidase gene of *O. oeni* was cloned and expressed in *E. coli*. The enzyme displayed properties similar to the glucosidase from *L. brevis*. Both enzymes were able to hydrolyze glycosides extracted from Muskat wine.

**Keywords:** β-glucosidase; *Lactobacillus brevis*; *Oenococcus oeni*; wine aroma
Growth Kinetics of Biopigment Production by Thai Isolated *Monascus purpureus* in a Stirred Tank Bioreactor

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*Monascus purpureus* is one of the biopigment producing fungi which its pigment can be applied in many biotechnological and food industries. The growth kinetics of biopigment productions were investigated in the liquid medium fermentation in a 5 L stirred tank bioreactor at 30 °C, pH 7 for 8 days with 100 rpm/min agitation and 20 lb/in² aeration. Thai isolated *Monascus purpureus* TISTR 3002, 3180, 3090 and 3385 were studied for color production with the nephrotoxic activity as the citrinin evaluation. Scanning spectra of the biopigment production revealed that each strain had the maximum absorptions of yellow, orange and red color at different wavelengths. TISTR 3002 produced lower amount of color than 3180 approximately 2.80, 1.37 and 2.62 times. Total color difference value between these two strains was 21.94 UA. Growth kinetics showed that the specific product formation of TISTR 3180 in the pigment productions were higher than these of 3002, which were 2.19, 6.38 and 13.12 times. For the specific substrate consumption of TISTR 3002, value was found higher than that of 3180 about 10%. Pigment production revealed that 3090 produced colors lower than that of 3385 equivalent to 1.41, 1.03 and 1.12 times. The total color difference between two strains was 47.20 UA. Growth kinetic exhibited that the specific substrate consumption of TISTR 3090 was lower than 3385 about 27%. The specific product formations of both strains were relatively the same with the values of 1.01, 0.83 and 1.25 times. TISTR 3385 had the higher pigment productivity than 3090 equivalent to 1.65, 2.06 and 1.89 times, respectively. HPLC results showed that all strains were not produced citrinin. L*, a* and b* values of the CIELAB color system for the derivative pigments of all strains were also reported.

**Keywords** *Monascus purpureus*, Biopigment, Growth kinetics, Citrinin

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Haloarchaenal Fermentation Technology for Recovery of Nutrients from Fish-waste

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In the coastal state of Goa - India, large fishes such as kingfish, tuna, shark, catfish and ribbon fish are sold in local markets as fish-fillets or fish-slices. The process generates fish-waste (FW) that includes substantial quantities of fish-flesh adhering to skeletal parts, skin, heads, bones besides entrails and off-cuts. Although FW is a rich source of edible nutrients, often times, can only be used as manure, because of its liability to autoysis and putrefaction, at tropical temperatures. This paper describes a microbial fermentation process, for the conversion of raw fish waste by Haloarchaea, under high salt conditions.

Seven different Halobacteria, isolated from “Salt pans” of Goa- India and having proteolytic activity at, 15 to 25% of crude – salt, at ambient temperature (28-30 °C) were used as a consortium to ferment raw solid fish - waste (FW) to “fish-sauce”, which yielded “Fish-paste” and “Fish-hydrolysate” in ratio of (1:3.5 w/v).

The deep brown liquid - fish - hydrolysate, at pH 5, had a pleasant fruity aroma and was rich in essential amino acids, phenylalanine < tryptophan < valine < tyrosine, and non-essential amino acids, namely glutamic acid, asparagine, proline and taurine. Substantial quantities of fattyacids such as undecylenic acid, linolenic acid, linoleic acid, arachidic acid, docosahexaenoic acid, teriric acid and behenolic were also present.

The devised process of “Haloarchaea fermentation of Fish waste” is a user-friendly, hygienically clean, viable microbial-biotechnology for release and recovery of beneficial nutrients as fish-hydrolysate which in turn could be used in preparation of “Functional foods”.

**Key words**: Fish –waste, Haloarchae, Fermentation, Hydrolysate, Functional –foods

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Heat stress adaptation of *Escherichia coli* under dynamic conditions: effect of inoculum size and heating rate

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**Introduction and objectives.** Temperature influences largely microbial behaviour and is one of the most studied abiotic stress factors. Several studies focus on the bacterial thermostolerance induced by temperature. Van Derlinden et al. (2008) observed disturbed growth curves of *E. coli* K12 MG1655 at temperatures near the maximum temperature of growth (T\text{max}). After a first growth and inactivation phase, the cell concentration increases again. This phenomenon can be explained by considering two subpopulations, i.e., a thermoresistant and a thermosensitive one. To further study the behaviour of *E. coli* at temperatures around T\text{max} experiments are performed in a computer controlled bioreactor under dynamic temperature conditions. By varying the initial cell concentration and the slope of a linearly increasing temperature profile crossing T\text{max}, the microbial adaptation to heat stress is studied.

**Results.** Depending on the initial cell concentration and the slope of the temperature profile, the evolution of the microbial population shows three, four or five subsequent phases, i.e., an exponential growth phase, a stationary phase (when the cell concentration reaches its maximum value or when growth stops due to the high temperature), an inactivation phase, a secondary growth phase and a secondary stationary phase (see Figure 1).

![Figure 1: Evolution of E. coli in dynamic temperature conditions. The temperature profile has an initial temperature of 42°C, a slope of 1°C/h (left) and 4°C/h (right) and a final temperature of 65.2°C. (--) depicts the detection limit.](image)

**Conclusion.** The acquisition of temperature resistance by *E. coli* is reflected in three aspects of the experimental results. (1) The exponential growth ends when the maximal growth temperature, T\text{max}, is reached. The steeper the profile, the higher T\text{max} except when the maximum cell concentration is attained first. (2) An analogous conclusion can be formulated for the influence of the slope of the temperature profile on the inactivation temperature, i.e., a steeper slope results in a higher temperature of inactivation. (3) Consistent with the findings of Van Derlinden et al. (2008, 2009), a secondary growth phase is observed after the inactivation of the sensitive cell population, picturing the growth of a resistant cell population. Figure 1 (right) shows that *E. coli* stills grows at the highest temperature of the profile, i.e., 65.2°C, a temperature significantly higher than the expected T\text{max}.

The unexpected influence of the temperature profile on the temperature resistance of *E. coli* and the observed growth at a temperature of 65.2°C are very important for food safety and temperature treatments of microorganisms.

**Acknowledgements.** Research supported in part by KULeuven-BOF Projects OT/09/25 and EF/05/006 OPTEC Optimization in Engineering, and by the Belgian Program on Interuniversity Poles of Attraction, initiated by the Belgian Federal Science Policy Office. Iris Cornet is supported by the Artesis University College of Antwerp.

**References**


**Keywords** *Escherichia coli*, temperature; heat resistant; stress adaptation

Heterogeneity of heat resistant proteases from milk spoiling *Pseudomonas* spp.

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Proteolytic psychrotolerant bacteria, predominantly *Pseudomonas* spp. still cause significant losses for the dairy industry, due to the production of thermostable proteases in raw milk. A polyphasic identification approach led to the identification of *P. fragi* and *P. lundensis* as predominant milk spoilers (53% of 103 proteolytic isolates) in 89 Belgian raw milk samples. In general, the *aprX* gene, encoding an alkaline metalloprotease, is believed to be the key feature in proteolytic spoilage by pseudomonads. However, up to now the presence of the *aprX* gene was not yet demonstrated in *P. fragi* and *P. lundensis*. To evaluate the presence of the *aprX* gene within *Pseudomonas* members, 29 *Pseudomonas* milk strains and 26 *Pseudomonas* reference strains were screened for the presence of the *aprX* gene in their genome. Although zymogram analysis revealed that all *Pseudomonas* milk strains produced heat stable proteases with a similar molecular weight, analysis of the protease sequences on the DNA and the protein level revealed a high heterogeneity within these heat stable *Pseudomonas* proteases. The obtained data revealed some interesting features for the development of identification and detection methodologies for pseudomonads in milk. Moreover, our data revealed that interspecies variations within the *aprX* gene could contribute to a molecular classification framework for *Pseudomonas* spp., in particular within the heterogeneous *Pseudomonas* fluorescens group.
Host responses to *Saccharomyces cerevisiae* isolates: new criteria to select safe strains

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Saccharomyces cerevisiae strains are the most widely used yeasts in industrial food and beverage production, traditionally regarded as absolutely safe and even *S. boulardii*, considered as a subtype of *S. cerevisiae*, has been described as a biotherapeutic agent in the prevention and treatment of bacterial diarrhoea and colitis in humans. Moreover, *S. cerevisiae* strains can colonize the respiratory, gastrointestinal and genitourinary tracts in a saprophytic way without causing disease; however, in the last decades there is increasing evidence indicating that *S. cerevisiae* is responsible for superficial diseases, that are not life-threatening, although in severely immuno-compromised individuals serious systemic infection may occur [1]. Using molecular techniques to characterize *S. cerevisiae* clinical isolates, both *S. boulardii* and baker’s yeasts were identified; the link between a subset of clinical isolates and probiotic or baking *S. cerevisiae* strains has been confirmed by phylogenetics and metabolomics [2-4].

The association between clinical isolates and non-clinical strains points out to food and probiotic/biotherapeutic strains from *Saccharomyces* species as a possible origin of human systemic infections [5]. Therefore, as the reports of *S. cerevisiae* infections are increasing steadily, this yeast species is now considered as an emerging low virulence opportunistic pathogen rather than a non-pathogenic yeast.

We have studied the virulence and host responses to several clinical and non-clinical *Saccharomyces cerevisiae* isolates: two vaginal isolates (60, 61), one isolate from faeces (29), a brewer’s yeast isolate used in dietetics (D14), one *S. boulardii* isolate from a commercial probiotic product, and a reference natural wine yeast (ECT 10431) [6].

Hematogenously disseminated infection in a mouse model demonstrated that four isolates (all, except 20 and 10431) were able to colonize preferentially the brain, as well as kidney and spleen to a lesser extent, of immunocompetent mice. *In vitro* adhesion assays to epithelial and endothelial cell lines also showed an increased adherence ability of strains 60, 61, D14 and *S. boulardii*. *In vitro* cytolysis production assays by RAW 264.7 murine macrophages challenged with yeasts showed a relative increased production of TNF-α in response to the 20 and 10431 strains; viability of RAW cells after coculture was similar in all cases (2-5% non-viable cells) except for 60 strain (11% non-viable cells). *In vitro* phagocytosis assays of yeasts by RAW cells showed that two isolates (D14 and particularly *S. boulardii*) were engulfed less efficiently. These results point out that *S. cerevisiae* isolates, from both clinical and non-clinical (dietetic and probiotic) origin, may vary in the expression of putative virulence factors contributing to their ability to develop the infectious process.

In conclusion, our results support the notion that clinical and non-clinical (dietetic and probiotic) *S. cerevisiae* isolates may share some properties in their relationship with the host (such as adhesion to host tissues, resistance to phagocytosis and the ability to modulate the proinflammatory response) that contribute to the infectious process [6]. These properties can be considered as new putative virulence factors that should be added to the already described (growth at 42 °C, pseudohyphal development, and production of proteases and phospholipases) [4,7]. Therefore, we propose that selection criteria for industrial strains should also include studies concerning host-pathogen interactions, such as virulence in animal models, adhesion to cell cultures, and *in vitro* cytolysis production and phagocytosis, in order to select totally safe strains.

References


Keywords: *Saccharomyces cerevisiae*; virulence; adhesion; proinflammatory cytokines; phagocytosis

Identification and Characterization of Yeasts Isolated from Oleic Ecosystems

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The aim of this study was to know the yeast biodiversity from olive (*Olea europaea* L.) fruits, olive paste and olive pomace from Arbequina and Cornicabra varieties of the region of Castilla-La Mancha (Spain). Yeasts were isolated from fruits randomly harvested at various olive groves. Olive paste and olive pomace (byproduct of this raw material process), were also collected in sterile flasks from different oil mill plants. 108 yeasts were isolated and identified by described as a biotherapeutic agent in the prevention and treatment of antibiotic-associated diarrhoea and colitis in means of comparison of polymerase chain reaction (PCR) amplicons of their 5.8S rRNA gene and internal transcribed spacers ITS1 and ITS2 (ITS1-5.8S-ITS2 region), followed by nucleotide sequence analysis. The results were compared to sequences held in public databases (BLAST). Later sequences obtained were deposited in the GenBank data library. Fourteen different species of yeast were identified, belonging to seven different genera (*Zygosaccharomyces*, *Pichia*, *Lachancea*, *Kuyveromyces*, *Saccharomyces*, *Candida*, *Torulaspora*). The most mainly-isolated species were *Pichia carpophila*, *Zygosaccharomyces fermentans* and *Pichia holstii*, followed by *Pichia mississippiensis*, *Lachancea sp., Klyveromyces thermotolerans* and *Saccharomyces rossini*. The activity of some interesting enzymes was also studied due to their biotechnological properties such as β-glucosidase, β-glucanase, carboxymethylcellulase, polygalacturonase, peroxidase and lipase. The majority of them showed β-glucanase, β-glucosidase and peroxidase activities, a few presented cellulase and poligalacturonase activities and the most significant fact, was that none of species had lipase activity.

Keywords: Yeast, olive, enzyme.
Inability to resuscitate viable-but-nonculturable cells of *Escherichia coli* and *Listeria monocytogenes*

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Viable-but-nonculturable (VBNC) cells were identified in 1982 and since then obtained a lot of attention from the scientific community. Today this is one of the most important areas of work in the food safety area. When in the VBNC state, bacteria are no longer culturable on conventional growth media, but the cells exhibit active metabolism, respiration, membrane integrity, gene transcription and present wall modifications that may be seen as a cell protection mechanism in adverse environmental conditions. Recognition of this state occurred initially in *Vibrio* spp. but currently is documented in a large number of species, mostly in gram-negative bacteria. Moreover, today there are evidences that a great amount of conditions usually taken as killing bacteria actually induce VBNC state. Among these conditions we can find those used as food hurdles, like extreme temperatures, low oxygen availability, high saline concentrations, extreme pH and low relative humidity.

This work started by induction of VBNC state in six strains of *Escherichia coli* and six *Listeria monocytogenes*, two internationally recognized food borne pathogens. Amongst the strains there are four clinical strains, five food strains and three isolated from food contact surfaces.

The VBNC state was induced by water, saline solution (0.9% sodium chloride), saline solution at pH 5, and 4 and 7% sodium chloride at 4°C and room temperature (totaling 10 different conditions). With these conditions we intended to simulate environments encountered by bacteria in food products, like acid pH, high salinity and in some special cases low nutrient content; saline solution provided us with a condition closer to the physiological one.

Periodically we tested the suspension for culturable cells in rich medium and in rich medium supplemented with an H₂O₂-degrading compound, to detect in this way cells susceptible to oxygen reactive species. When culturability was no longer obtained in any of the media viability was confirmed by Direct Viable Counts. At this point resuscitation experiments were carried on.

Resuscitation trails were promoted in a great number of conditions: rich medium diluted to different strengths, supernatant of late exponential phase cells in rich and minimal media diluted to different strengths, rich medium supplemented with ATTC® trace mineral supplement, minimal medium supplemented with amino acids, temperature upshift and heat shock. To exclude growth of some residual culturable cells that might be present in the suspension we followed two approaches: VBNC cells were incubated in resuscitation conditions with ciprofloxacin, in a concentration higher to minimal inhibitory concentration, to prevent growth; Micro Most Probable Number Method was used with the different resuscitation media.

In none of these conditions resuscitation was observed. This suggest that the exact combination of factors that lead to resuscitation of VBNC cells of *E. coli* and *L. monocytogenes* induced by conditions similar to those employed as food hurdles were not achieved in the performed experiments. If the stimulus that leads to resuscitation is unique, like one single molecule, that molecule was not present or not in the correct amount in the media used. If the stimulus that leads to resuscitation of these cells is a composed one (like a combination of molecules or abiotic variables), that could guaranty the cells that resuscitation only occur when the best conditions for survival of the population are achieved, that combination was not present in the conditions tested. More resuscitation conditions are currently being tested.

Keywords: viable-but-nonculturable; resuscitation; *Escherichia coli*; *Listeria monocytogenes*

Influence of Bacteriocin producing *Lactobacillus casei* RN 78 in Growth Control of *L.monocytogenes* in Experimental Cheese samples

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In this study, anti-listerial effect of a previously isolated *Lactobacillus casei* RN 78 strain and freeze dried fractions of its partially purified bacteriocin (Fd-PPL Lactocin RN 78) was evaluated in experimental cheese samples stored at two different temperatures (4 and 35°C). With the addition of 6400 AU/g of Fd-PPL fractions to the cheese samples the initial concentration of 6.81 +/- 0.06 log CFU/g of *Listeria monocytogenes* was reduced up to 0.71 +/- 0.01 log CFU/ml. In comparison to the bacteriocin fraction (Fd-PPL), addition of 10⁷ CFU/ml of the bacteriocinogenic strain (LP and LBP) in the cheese samples stored at 35°C, appeared more effective in inhibiting and controlling the growth of mentioned pathogen. The viability of the sensitive cells decreased sharply (0.21 ± 0.02 log CFU/ml) in the presence of the live bacterial cells of *L.casei* RN 78. The effect was more pronounced after 24 hrs of incubation and high levels of bacteriocin activity (12800 AU/ml) was seen in these cheese samples. Moreover, the pH of these cheese samples was significantly effected by the presence of producer viable cells and very comparatively lower (4.8-5.0) compared to the other cheese samples which did not include the producer strain. A synergic bactericidal effect of Lactocin RN 78 and 3% NaCl in BNP cheese samples was observed, and in combination they were able to reduce the *L.monocytogenes* population to 0.59 log CFU/ml within 90 days of incubation at 35°C. The antibacterial effect of Lactocin was more pronounced in samples stored at 4°C then at 35°C. In contrast, the producer strain was more effective in control of *L monocytogenes* in cheese samples during storage at 35°C.

The texture of the experimental cheese samples including odor, color and consistency in different batches were also recorded through out the study.

Keywords: *Lactobacillus casei*; RN 78; *Listeria monocytogenes*
Influence of different photoperiods on the incidence of *Aspergillus niger* in coffee beans stored

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To ensure the quality of coffee is essential to identify the critical factors that can influence it, among which, the conditions that lead to the proliferation of *Aspergillus niger* when the grains are stored, since this fungus can negatively influence the characteristics crucial to their marketing. This study aimed to investigate the influence of different times of light exposure on the proliferation of *Aspergillus niger* in coffee beans stored for a period of nine months. For both coffee beans with and without inoculation of *Aspergillus niger* were stored in wooden boxes for the control of photoperiod (dark, 12 hours and 24 hours of light) in order similarity to the conditions of storage of coffee. The weight of coffee beans was checked every month and the samples were analyzed for the presence of the fungus. The pH was not significantly different in the coffee beans inoculated with *Aspergillus niger*, indicating that the fungus had little influence this parameter, unlike acidity, where the highest incidence of this species had a significant impact. The grain moisture was similar in all samples, as the ideal storage (10 to 13%). Thus, photoperiod with light and longer time (24h) reflected a greater proliferation of *Aspergillus niger* in coffee interfering therefore in their quality, as it resulted in higher levels of phenolic compounds and a drastic reduction of color. Thus, it is concluded that light and photoperiods with longer time (24h) can directly affect the quality of coffee beans, since they allow greater proliferation of *Aspergillus niger* and consequently higher levels of phenolic compounds and a drastic reduction of its coloring.


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Influence of pesticides on the growth kinetics of yeasts used as starter cultures in green table olives

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Lactic acid bacteria and yeast are essential in the fermentation stage of different kinds of green table olives. The role of lactic acid bacteria can be partially replaced by a rapid acidification with lactic or tartaric acids in elaborating seasoned green table olives, with the fermentation mainly carried out by yeasts. The presence of pesticide residues in the olives and brine may affect the activity of yeast fermentation. Any interruption or delay in this process can alter the end product’s qualitative and quantitative characteristics. The aim of the present work was to study the growth of potential starter culture *Pichia anomala* strains for seasoned green table olives in synthetic brines containing different pesticides as the main carbon source. For this purpose a synthetic brine was prepared which contained 0.5% (w/v) calcium lactate, 7% (w/v) NaCl, 1% (w/v) Yeast Nitrogen Base, together with different concentrations of several pesticides. An automated turbidimeter Bioscreen C Microbiology Reader was used in an in vitro experiment to monitor the growth of yeast isolates in the presence and absence of pesticides by reading the optical density (OD) at 600 nm at regular intervals. In a pilot plant experiment, survivals of the yeast strains were examined by plating onto their specific media at 0, 1, 5, 21, 35, 70, and 90 days of fermentation. Although the growth of yeast strains was affected by the pesticides in the in vitro experiment, there was no such effect in the pilot plant experiment. This suggests that the pesticides are partially adsorbed by olive constituents, thus minimizing their effect on the yeast growth.

Keywords: Olives; yeast; pesticides; biodegradation
Interactions between Saccharomyces cerevisiae and non-Saccharomyces wine-related strains: inhibitory activity of small peptides produced by S. cerevisiae CCMI 885

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During spontaneous wine fermentations non-Saccharomyces (NS) yeast strains begin to die off after the first days of alcoholic fermentation leaving way to the more fermentative S. cerevisiae strains to complete the vinification process (Egli et al., 1998; Fleet, 2003). This behaviour has been always attributed to the weaker capacity of these yeast strains to withstand the increasingly stressful conditions -high ethanol and organic acid concentrations, low pH values, scarce oxygen availability or nutrient limitations-, occurring during wine fermentations (Fleet and Heard, 1993). However, previous work (Pérez-Nevado et al., 2006) carried out with two Hanseniaspora strains (H. guilliermondii and H. woodii) showed that early death of these two NS strains during mixed fermentations with S. cerevisiae (Sc) was not primarily induced by ethanol, nutrient or oxygen depletion, low pH values or classical killer toxins, but rather by other unknown toxic compounds. In the present study we investigated the nature of those toxic compounds and evaluated their inhibitory effect on the growth of some NS wine-related strains.

Firstly, the capacity of Sc supernatants to induce death of H. guilliermondii (Hg), Klyuyveromyces marxianus (Km), K. thermotolerans (Kt) and Torulaspora delbrueckii (Td) was determined and showed that all NS strains died on those supernatants. Then, protease treatments of death-inducing supernatants, either with pepsin or with a mixture of trypsin plus alkaline protease, revealed the proteinaceous nature of the toxic compounds. The analysis of the protein pattern of mixed (Sc/NS) supernatants on Tricine SDS-PAGE showed that Sc CCMI 885 secretes small peptides (<10 kDa) that were detected only when death of Hg was already established. Death-inducing supernatants were ultrafiltrated by 10 and 2 kDa membranes, respectively, and lower than 2 and 10 kDa protein fractions tested regarding their ability to induce death of Hg. Results showed that the (2-10) kDa protein fraction of Sc supernatants exhibited inhibitory activity against Hg. These (2-10) kDa protein fraction, containing three peptides with apparent molecular weight (MW) of 4.0, 4.5 and 6.0 kDa, was concentrated using membrane ultrafiltration systems and its antimicrobial activity tested against strains of Hg, Km, Kt and Td. Under the growth conditions used for these tests, the small peptides inhibited the growth of all NS strains and induced death of Km.

Keywords: antimicrobial peptides; yeast-yeast interactions; wine fermentations; yeast population dynamic;

References:
Isolation and characterisation of exopolysaccharide producing lactic acid bacteria

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Exopolysaccharides (EPS) synthesized by lactic acid bacteria (LAB) are of economic importance because they can impart functional properties on foods and may confer health benefits to the consumer. For example, β-glucan, which can be produced by some LAB, is suggested to have the ability to lower cholesterol. Claims have also been made that other EPS may have immunostimulatory activity and antimicrobial effects. In this study a bank of 3500 LAB strains from milk, beer and grain sources, were screened for EPS production, using Congo-Red and mMRS agar (containing 5 to 10% sucrose or glucose). Based on their EPS-producing ability, an initial group of 600 EPS producing LAB was identified and from these a sub-group, containing LAB which produced the highest levels of EPS, was further characterized. This sub-group was found to include strains of Lactobacillus rhamnosus, Lactobacillus casei, Lactobacillus brevis, Lactobacillus curvatus, Lactobacillus plantarum, Lactobacillus pentosus, Lactococcus fermentum, Leuconostoc citreum, Leuconostoc mesenteroides, Lactococcus lactis, Weisella cibaria and Weisella confusa. When the initial bank of 600 EPS producers was screened for genes responsible for β-glucan production, using primers based on glucosyltransferase genes (gtf) from known elaborators of β-glucan, the strains Lactobacillus brevis, Lactococcus lactis and Leuconostoc citreum gave a positive amplicon. The bank of EPS producing LAB generated in this study represents a source of potentially valuable strains for use in the development of functional foods.

Isolation and characterization of cysteine biosynthetic gene in \textit{Lactobacillus casei} encoding cysteine lyase and synthase activity

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Lactic acid bacteria are widely used as starter and nonstarter cultures in the dairy industry. In cheese they play an essential role in the degradation of casein-producing amino acids which are converted to essential flavour compounds mainly by enzymatic pathways. Metabolism of sulfur-containing amino acids in \textit{Lactobacillus casei} has not been extensively studied. Recently, we observed that several \textit{Lactobacillus casei} strains isolated from Gruyère cheese could grow in a chemical defined medium containing methionine as the sole sulfur source (Irmler \textit{et al.}, 2008). This indicates that cysteine biosynthesis must occur. There are no data about cysteine biosynthesis pathway in \textit{Lactobacillus casei} and our first goal was the characterization of the \textit{cysK} gene potentially encoding cysteine synthase. The \textit{cysK} gene was cloned from the \textit{Lactobacillus casei} FAM 18110 strain isolated from Swiss Gruyère cheese and recombinant protein was overproduced in \textit{E.coli}. It was shown that recombinant enzyme is active in synthesis of cysteine from O-acetylserine and sodium sulfide and on the other hand in production of hydrogen sulfide from L-cysteine. Km and Vmax values of the enzyme for both activities were determined. The role of \textit{cysK} gene product in role in cysteine biosynthesis was confirmed in complementation experiments with the cysteine auxotroph mutant strain, \textit{E.coli} NK3.
Isolation and identification of molds associated with table olives

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Mold growth on table olives may be implicated in different problems of spoilage, one of which is related to their ability to produce mycotoxins. In this study twelve mold strains were isolated during the processing of green table olives. These molds were identified by different methods: studying their macroscopic and microscopic characteristics; according to their secondary profiles of metabolites analyzed by micellar electrokinetic capillary chromatography (MECC); and using internal transcribed spacer regions 1 and 2 (ITS 1-5.8S-ITS 2). The results showed that four of the isolates belong to Penicillium expansum, one to Penicillium glabrum, three to Aspergillus flavus; two to Fusarium solani; one to Beauveria bassiana and two were identified as the yeast-like fungus, Galactomyces geotricum. The ability of the isolates to synthesize mycotoxins on malt extract agar was investigated by MECC. Four strains produced a total of three mycotoxins: three strains, identified as Penicillium expansum, produced roquefortine, and one, identified as Aspergillus flavus, produced two mycotoxins, cyclopiazonic acid and aflatoxin B₁.

Keywords Olives; mold; identification; micellar electrophoresis; mycotoxins

Killer toxin of Pichia anomala strains isolated from olive brine and active against human pathogens

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Killer activity has been shown in many P. anomala isolates; the killer system described in this species shows toxic activity against a wide variety of nonrelated microorganisms, such as hyphomycetes and bacteria, including important opportunistic pathogens, such as Candida albicans. Because the killer phenotype could not be cured by the application of cycloheximide, ethidium bromide or acridine orange, it was suggested that the killer genes are chromosomally located. In this study, 22 Pichia anomala strains, isolated from naturally fermented olive brine, showing high killer capacities against sensitive strain (strain 5×47) of Saccharomyces cerevisiae, were characterized according to the interactions among the isolates and prokaryotic and eukaryotic human pathogens (Candida albicans, Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Enterobacter aerogenes, Enterococcus faecalis) and examined for detection of genetic elements, which code for the killer character (dsRNA, ds linear DNA or a chromosome). Killing ability and resistance among P. anomala isolates and pathogenic C. albicans and S. cerevisiae were determined on YEPD-MB agar (1.0% yeast extract, 2.0% peptone, 2.0% glucose, 0.1M citrate-phosphate buffer pH 4.5, 0.01% methylene blue and 2.0% agar). The strains with killer activity appeared surrounded by a clear zone, surrounded by a blue precipitated halo indicative of cellular death. Killing assay against bacterial clinical isolates was carried out in Trypticase Soy Agar; in this case antimicrobial activity was recorded as growth free inhibition zones. Nucleic acids were extracted from P. anomala isolates; RNA/DNA samples were treated with Nuclease SI, DNasel, RNaseH and RNaseA buffered at different ionic strength. The killer yeast strains of P. anomala showed considerable activity against bacterial pathogens, both gram positive and gram negative. In fact, 12 strains of P. anomala have shown antimicrobial activity against Staph. aureus and Staph. hominis and 14 strains were active against Enterobacter aerogenes. These result confirmed previous studies, which suggested a widespread antimicrobial activity of yeast killer toxin against gram positive but also demonstrate action against gram negative bacteria, probably due to the cell wall composition and permeability among different species. Regarding yeast clinical isolates, no killer activity was detected against Candida albicans and Cryptococcus sp., while it was effective against S. cerevisiae.

At a molecular level, all K+ isolates of P. anomala do not display any EGEs, suggesting that the phenotype is encoded in the genome of the cells.

Keywords Pichia anomala, killer toxin, clinical yeast and bacterial isolates; killer phenotype encoding
Lactic acid bacteria from wines from Ribeira Sacra (Spain): Isolation, identification and characterization of some oenological properties

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The appellation of origin (DO) “Ribeira Sacra” is a young DO from Galicia (Spain) in which red wines of high quality are produced from “Mencía” grapes. As red wines, the malolactic fermentation (MLF), always necessary, is usually carried out spontaneously by the autochtonous lactic acid bacteria (LAB), which characterize the organoleptic properties of the wines from every zone.

The objectives of the present study were: the establishment of a wide collection of LAB isolated from this DO, including representative LAB from every zone, as well as the characterization of the isolated LAB and the conservation of the strains with oenological properties of interest in order to provide them to the winemakers of Galicia.

To achieve a large and diversified collection of LAB, two vintages (2007-2008) and four wine-cellar were selected, thus the variability due to differences in the climate and the different five zones of the DO were covered.

The samples were taken from must in 2008 and after alcoholic fermentation in 2007 and 2008. Appropriate dilutions of must and wine were plated on Petri dishes containing MRSCNT (MRS + 0.5g/l of L-Cysteine + 50mg/l of Nystatin + 50ml/l of tomato juice) and incubated at 30ºC during 5 days in anaerobic jars. 20-40 colonies from every sample were plated in patches on MRSCNT and incubated in anaerobic jars during 4 days at 30ºC. Using the grown patches, tubes containing MRSMT (MRS + 5g/l of DL-Malic acid + 50ml/l of tomato juice) were inoculated and incubated in order to stock the isolates with glycerol, identify the species and analyze their putative functions of oenological interest and their content in plasmids.

To identify the species, biochemical tests, API straps, and PCR and Multiplex-RAPD techniques (Araque et al, 2009; Beneduce et al, 2004; López et al, 2008; Reguant and Bordons, 2003) were used. Plasmid analysis was made according to O’Sullivan and Klaenhammer (1993). B-glucosidase and tannase activities were assayed as per Barbagallo et al (2004) and Vaqueró et al (2004) respectively.

The results obtained showed that the diversity of the population of LAB in the musts and wines from the “Ribeira Sacra” is influenced by all factors analyzed in this work: vintage, wine-cellar (zone) and addition of sulfite. The presence of Lactobacillus at high pH and high alcoholic degree is in contrast with the presence of Oenococcus at lower pH and alcoholic degree. This diversity, wide in must, becomes narrower during the MLF in which Pediococcus and Leuconostoc disappear favouring the presence of Lactobacillus (mainly Lb. plantarum) and O. oeni. The abundance of strains with different characteristics provides us a wide and diversified collection of LAB.

Microbial conversion of major ginsenosides to pharmaceutically active minor ginsenoside C-K by Dyella sp. QGC-49

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Ginseng saponin, the most important secondary metabolite of ginseng, has various pharmacological activities. To obtain minor saponins from major ginsenoside using microbial biotransformation, many soil bacteria which has strong β–glucosidase activity were screened. One bacterial strain QGC-45 could convert ginsenoside Rb1, Rb2, Rc, Rd to the active metabolites C-K via F2, C-Y, C-Mc, respectively. Phylogenetically, it was found to belong to genus Dyella and was very closely related with Mucilaginibacter koreensis BB4 (93.2% similarity based on 16S rRNA sequence). Enzymatic production of C-K occurred by consecutive hydrolyses of the terminal glucopyranosyl, arabinopyranosyl, arabinofuranosyl moieties at the C-20 carbon and hydrolyses of the terminal and inner C-3 carbon of ginsenoside Rb1, Rb2, Rc showing the biotransformation pathway: Rb1 → Rd → F2 → C-K; Rb2 → C-O → C-Y → C-K; Rc → C-Mc1 → C-Mc → C-K, respectively.

Keywords: wine, lactic acid bacteria, B-glucosidase, tannase

References

Keywords: ginseng, ginsenoside, biotransformation, compound-K
Microbiological Changes in Cheese of Algarvian Goat Breed during Ripening

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A traditional cheese from Algarve region (Portugal) is produced with raw milk from algarvian goat breed and is coagulated by aqueous extracts from cardoon flowers (Cynara cardunculus). These cheeses are produced under artisanal conditions, and during the processing procedures several contaminations may occur, which could influence their quality during ripening.

The main goal of this work was to monitor the cheese microbial quality along a ripening period of sixty days.

Samples were prepared for microbiological analysis according to NP1829 (1982). Aerobic bacteria, coliforms, yeasts and moulds were enumerated according to NP1995 (1982), NP3788 (1990) and NP3277-1 (1987), respectively. Presumptive lactobacilli were incubated on Rogosa agar (RA) (Biokar) at 36±1 ºC, during 72 h and presumptive lactococci were incubated on M17 agar (Biokar) at 30±1 ºC, during 48 h. All assays were done in triplicate and expressed as colony-forming units per gram (cfu/g) of cheese.

During cheese ripening, the total viable microorganisms showed no considerable differences, presenting values of 9.02 log cfu/g after 60 d of ripening. The initial increase of one log should be due to the Lactic Acid Bacteria (LAB) growth, but it was rapidly compensated by the decrease of coliform bacteria.

The enumeration of presumptive lactobacilli reached a maximum after 42 days of ripening (9.18 log cfu/g) and for presumptive lactococci at 14 days (9.86 log cfu/g). A slightly decrease was observed after that. Nevertheless, the concentrations of both lactobacilli and lactococci reached similar levels after 28 days of cheese ripening.

Total coliforms reached maximum concentrations values after 14 days of ripening, decreasing thereafter gradually by about 4 log units until 28 days of ripening. After this period, their concentrations become approximately constant.

Facal coliforms started from a maximum of 3.98±0.65 log cfu/g, at the production day and reached zero value by the 21 days of ripening.

Other microbial groups like moulds and yeasts reached maximum values at 14 days of ripening (7.70 log cfu/g) and then decreased at different rates until the end of the ripening period.

These results suggested that the lactic acid produced by the LAB in the beginning of the ripening period contributed to the decrease of the remaining microbiota, including the inhibition of the facal coliforms, which can contribute to the safety of the ripened cheese.

Keywords: goat cheese, microbiology, ripening

Microbiological control of wines from Denomination of Origin Rías Baixas in Galicia (NW Spain)

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Microorganisms play an essential role in wine elaboration. Thus, yeasts are responsible for alcoholic fermentation and lactic acid bacteria are involved in malolactic fermentation. However, when fermentation processes are finished, these microorganisms have to be eliminated during the wine maturation period by different procedures to avoid wine spoilage due to undesirable proliferation of yeasts and/or bacteria. Despite the importance of microbiological growth in wine stability not many studies have been published on this subject. Moreover, maximum levels of microbial contamination in wines have not been legislated by the Office International de la Vigne et du Vin (OIV).

In this work we exposed the microbiological evaluation of wines from 10 different wineries within Denomination of Origin Rías Baixas in Galicia (NW Spain). The study includes samples from bottled wines and samples of wines during storage in tanks before bottling for each winery.

Wine samples (100 ml) were filtered through out a 0.45 μm filter using a MilliflexTM filtration system from Millipore. The filter was placed on a WL nutrient media plate and incubated at 28ºC until visible colonies appear. Those plates presenting microbial growth were used for viable cells count and to isolate colonies for further analysis.

A total of 35 samples were processed. The results showed that 18 of them (51%), including all the bottled wines examined, did not contain visible microorganisms. The remaining samples presented a variable number of cells ranging from only 9 cell in 100 ml to concentrations of 10³ (cells/mL) or higher. The microorganisms found were mainly yeasts, although 4 samples presented also bacteria growth.

The identification of yeast isolates revealed that they belonged to at least four different species: *Saccharomyces cerevisiae* (found in 5 wines), *Pichia membraniformis* (isolated in 3 wines), *Zygosaccharomyces bailii* (found in 8 wine samples), and *Debaryomyces hansenii* (only present in one sample). These yeasts have been previously reported as common population of wineries during winemaking and wine storage.

Taking all the results together, it can be concluded that commercial wines from Denomination of Origin Rías Baixas show acceptable levels of microorganisms. The absence of yeasts and bacteria in bottled wines guarantees their microbial stability. In addition, the low number of yeast cells in bulk wines is within values considered to keep the wine clear. Nevertheless, the presence of *Z. bailii* in a high number of samples should be taken under consideration because this species has been described to be resistant to chemical preservatives used in winemaking.

Keywords: wine, microbiological control, yeast species identification
Microbiological risk assessment of *Staphylococcus aureus* in sandwich products consumed by airline passengers

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A microbiological risk assessment of *Staphylococcus aureus* in tuna (TBFS) and meat (MBFS) based filling sandwich products consumed by airline passengers were conducted to investigate the probability of health risk resulting from sandwich consumption. The risk assessment model was based on the results of the concentration of *S. aureus* in sandwich products and the rate of *S. aureus* contamination sampled in the airline catering from 2007-2008. Two different exposure models were proposed for TBFS and MBFS. Because of limited data and absence of human dose - response model on *S. aureus*, simulated dose - response from animal study was carried out with the assumption that conversion from animal to human is 1000. For each of these two sandwich groups, one hit dose-response model was used to estimate the probability of gastrointestinal illness as a function of the ingested dose using Monte Carlo simulation technique. The estimated amounts of contaminated TBFS and MBFS with *S. aureus* were found to be between 198 to 354 and 166 to 234 meals per year respectively. The estimated number of illness due to consumption of MBFS among airline passengers from a total of 703,320 passengers per year. These risk assessment models are important to the management in reviewing the critical factors such as effectiveness of temperature control and processing practice that have a great impact in preventing and reducing risk of *S. aureus* poisoning.

**Keywords:** Microbiological risk assessment, *Staphylococcus aureus*, sandwich products, airline passengers

Modulation of the gut microflora by dietary fibres and characterization of extracellular metabolites and fermentation products by GC-MS analyses

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Dietary fibres may act as prebiotics, i.e. stimulation of growth of health-promoting probiotic bacteria such as bifidobacteria and lactobacilli. Fermentation of carbohydrates by the gut microflora generates short-chain fatty acids (SCFA), of which particularly butyric acid is considered as beneficial for the health.

*In vitro* fermentations of fibres with human faeces have frequently been used for comparison of fibres with respect to the formation of SCFA, analysed by GC or HPLC. In contrast to techniques directed against analyses of specific compounds, the use of LC- and/or GC-MS will generate an overall metabolite profiling, a "metabolic footprint". Such data may provide valuable information about the function of the microbiota, as a supplement to information gained from DNA-analyses, telling which bacterial genera and species that are present.

A range of fibres have been screened by *in vitro* batch fermentations with faeces from infants (3-7 months age) and adults. The fibres include polysaccharides or selected fibre fractions, such as β-glucans, arabinoxylan and poly-uronic acids (pectin, alginat). The commercial prebiotic inulin was included for comparison. SCFA were monitored by HPLC-analyses, while a complete metabolite profiling was achieved by use of GC-MS. Independent of the faeces source, the highest amount of SCFA, with acetate as the dominating acid, was produced from inulin and a pectin fraction from white cabbage. Highest amounts of propionic and butyric acids were produced from inulin and barley fibres. In general, the fermentations with infant faeces gave a higher fraction of propionic acid and less acetic and butyric acid than faeces from adults. The GC-MS analyses revealed that monomers of the polysaccharides were detectable only for a short period early in the fermentation, and that the inoculum included significant amounts of amino acids, probably from dead bacteria. Some of the amino acids were consumed during the fermentation, and the relative production rates of the individual SCFAs varied dependent on the substrate being consumed.

Changes in the composition of the infant faeces microflora after growth on the fibres were characterized by establishment and analyses of a 16S rRNA clone library. Distinct differences in the development of the flora during the fermentations were observed. For instance, β-glucan and barley fibres stimulated *Bacteroides*, while the pectin from cabbage stimulated growth of *Lachnospiraceae*. Inulin promoted an increase in *Bifidobacterium*, a well-known effect of this prebiotic. Cultivable bacteria enriched on each of the fibres have been isolated and are further characterized with respect to fibre degradation and fermentation products.

The present results demonstrate interesting differences between the action of dietary fibres, as well as the usefulness of more extensive analyses of substrate consumption and product formation.

**Keywords:** gut microflora; *in vitro* fermentation; GC-MS analyses
Molecular and convencional identification and caracterization by ARDRA and DGGE of the microbiota associated to semi-dry coffee (Coffeea arabica L.)

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Bacteria, yeast and filamentous fungi are isolated during all the stages of coffee processing. Thirteen samples of Coffea arabica L. were collected during different processing stages of semi-dry coffee from a farm in the South of Minas Gerais. The isolated bacteria and yeasts were identified by Amplified Ribosomal DNA Restriction Analysis (ARDRA) and sequence analysis of the 16-23S of the rDNA (yeasts). The filamentous fungi were identified by analyses of macro and microscopic characteristics of the colonies. Denaturing gradient gel electrophoresis (DGGE) of the product of PCR of the rDNA 18S and rDNA 16S were carried out to analyze the yeast and bacteria communities. Bacteria, yeast and filamentous fungi counts were in the order of 4.7 x 10^10 to 1 x 10^11 CFU/g, 2.3 x 10^8 to 7.5 x 10^10 CFU/g, 1 x 10^5 to 5.5 x 10^7 CFU/g, respectively. Using the technique ARDRA of the 16-23S region of the rDNA, 16 distinct restriction fragment patterns, corresponding to 16 different bacterial species were obtained. Bacillus subtilis, Escherichia coli, Enterobacter agglomerans, Bacillus cereus and Klebsiella pneumoniae were the predominant bacteria during the coffee processing. Lactococcus lactis, Serratia sp., Acinetobacter spp and Bacillus megaterium were isolated in culture medium, but not detected by DGGE analysis of the same samples. All of the species detected by DGGE were also isolated for cultivation, except for the nonculturable bacteria. The method ARDRA of the ITS1-5S-58S region of the rDNA allowed 15 distinct restriction fragment patterns corresponding to 14 different yeast species. Pichia anomala was present in all the samples, with counts to the order of 10^10 to 10^11 CFU/g. Torulaspora delbrueckii and Rhodotorula mucilaginosa were also predominant yeasts during the coffee processing. Some yeast species such as Candida amoldi, C. fukuyamaensis, Pichia caribbica, C. membranifaciens, Saccharomyces bayanus and Arxula sp. were isolated in culture media but not detected in the DGGE analyses of the same samples. All of the yeasts species detected by DGGE were also isolated in the culture media. Among the filamentous fungi identified, the genus Aspergillus was of higher incidence followed the genera Penicillium sp., Fusarium sp. and Cladosporium. There was a good correlation among the species found by isolation in culture medium and sequencing and the DGGE profiles obtained, for bacteria, as well as yeasts, however the molecular techniques need to be associated to the traditional techniques in order to obtain better characterization of the microbiota present in coffee processing.

Financial support: FAPESMG/CAPES/CNPq

Keywords: coffee processing, approach polyphasic, microbiota, fermentation

Molecular characterization and biodiversity of Saccharomyces cerevisiae in spontaneous fermentation in D. O. “Condado de Huelva” (Southern Spain)

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Traditionally, wine has been produced by the natural fermentation of grape juice carried out by yeasts present in the grapes and winery equipment. This spontaneous fermentation is a complex process characterized by the presence of a large number of different yeast genera and species. Yeast species with low fermentative activity, such as Hanseniaspora (Kloëckera), Candida and Metschnikowia, are dominant in the fresh must and during the early phase of fermentation. By mid-fermentation these yeasts begin to decline and die off. Under these conditions, different strains of Saccharomyces cerevisiae become dominant and complete the process (Fleet and Heard, 1993). However, the number of species and their presence during fermentation depends on several factors, with subsequent wine quality variations from region to region and from one year to another. All this make the outcome of spontaneous fermentation difficult to predict (Maro et al., 2007). Thus, in the last decades, the use of commercial Saccharomyces strains as starters is becoming a common practice in most wine-producing regions. However, there is some controversy about this practise due to the lack of some desirable traits provided by natural or spontaneous fermentation (Fleet and Heard, 1993). On other hand, the maintance of biological patrimony is essential both to obtain starter strains that are able to fully develop the typical flavours and aromas of wines originating from different cultivars, and to ensure the conservation of gene pools of primary importance for the preservation of productive activities based on yeast-mediated processes (Maro et al., 2007).

In this context, we have studied the Saccharomyces population which participate in spontaneous fermentations in six different wineries belonging to the DO “Condado de Huelva”, in which active dry yeasts have never been used, during two successive vintages (2007 and 2008). Samples were taken at the middle and the end of fermentation, serially diluted and 0.1 ml aliquots were spread onto plates of YEPD agar plates and incubated at 28 ºC for 72 hours. For each sample, 30 colonies were randomly chosen and subjected to further studies. Isolates were identified by PCR-RFLP of the region encompassing the 5.8S rDNA gene and the ITS1 and ITS2 regions; PCR products were digested with the restrictases HinfI, HhaI and HaeIII (Guillamón et al. 1998). Strains of S. cerevisiae were differentiated by RFLP of mtDNA using HinfI (Querol et al., 1992).

Of a total of 577 yeast colonies isolated, 569 corresponded to Saccharomyces cerevisiae strains presenting 195 different mitochondrial restriction patterns thus evidencing a great biodiversity (34 %). The number of different patterns isolated in each winery varied from 12 to 75 revealing significant differences in the biodiversity degree among the wineries. Most of the characterized patterns were unique (137) and were isolated only in one of the vintages under study. Conversely, a great number of the strains isolated (47.3 %) matched to only 9 different patterns and some of them were isolated during both vintages and in several of the wineries, this indicating that they might be resident yeasts characteristic of this region. Additionally, a clear sequential substitution of Saccharomyces strains was observed between the mid and the final fermentation stages, with only 33 patterns present in both phases in any of the wineries.

Keywords: Saccharomyces, winery, biodiversity, succession

Acknowledgements: This study was supported by the project RM2007-00008-C02-00 from INIA (Ministerio de Educación y Ciencia de España)

References
Molecular identification of yeast species associated with green table olive production

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Worldwide table olive production reached 2,153,500 tones in the 2007/2008 season. The presence of yeasts is very common in this food, where they have an important influence on the organoleptic characteristics of the final product. The aim of this work was to isolate and identify the yeast population associated with fermentations of both directly brined and green Spanish-style olives. Three different cultivars (Gordal, Manzanilla and Aloreña) were used in the experiments. A total of 199 isolates, obtained along fermentation process, were identified by molecular methods such as RFLP analysis of the 5.8S-ITS rDNA region and sequencing of the D1/D2 domains of the 26S rDNA gene. The most important species related to directly brined Aloreña olives were Pichia membranaefaciens (33%), Candida diddensiae (30%) and Saccharomyces cerevisiae (30%), while for directly brined olives of Gordal and Manzanilla varieties were Pichia galeiformis (45%), Pichia anomala (30%) and Candida tropicalis (21%). In green Spanish-style Manzanilla and Gordal olives, the predominant yeast species were Debaryomyces etchelli (35%), Kluyveromyces lactis (20%), P. galeiformis (18%) and C. tropicalis (12%). Other species isolated from these processes but at lower frequencies were Candida boidinii, Debaryomyces Hansenii, Hanseniaspora guilliermondii, Issatchenkia orientalis and Torulaspora delbrueckii. The restriction analysis of mitochondrial DNA carried out with isolates from the S. cerevisiae and K. lactis populations showed 5 different profiles for S. cerevisiae. On the contrary, a single profile was reported for K. lactis. This survey describes the yeast biodiversity present in table olive production.

Keywords: table olives; yeasts, molecular methods; biodiversity

New antifungal bacteriocin synthesizing strains of Lactococcus lactis ssp. lactis as the perspective biopreservatives for protection of raw smoked sausages

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Fungal spoilage of food is a common and global phenomenon. In additional to economic losses, the potential productions of toxins by fungi are of particular health concern. The aim of this study was to isolate and identify antifungal lactococci and evaluate their potential in preventing fungal spoilage. In our experiments we used raw smoked sausages that were infected with fungi. The identification of this spoilage showed the presence of Aspergillus repens on the sausages. The traditional use of the dairy lactococci in various food fermentations, their “GRAS” status, non-pathogenicity, the ability to synthesis of bacteriocins can be safety used in food protections as a food biopreservatives. Screening of effective bacteriocin synthesizing strains of Lactococcus lactis as the perspective biopreservatives was performed. We used the raw milk and milk products from differed climatic regions and also powerful drinks of mixed lactic acid and alcoholic fermentation: kurunga, kumiss and Iranian drink “Dough” which were widely used by people to prevent diseases. The special interest was paid to isolates of lactococci with antagonistic activity. According to their morphological, cultural, physiological, biochemical properties and gene sequence of 16S rRNA they were identified as Lactococcus lactis ssp. lactis. Antimicrobial activity studies revealed differences between the strains to the effects on individual groups of microorganisms. The activities of these strains were also distinct from Nisinplin one (commercial preparation of the bacteriocin nisin). Nisin kills Gram-positive bacteria including spore forms and is not affected on fungi. Only nine of the selected 94 strains expressed a broad spectrum of activity including against moulds: Aspergillus, Fusarium, Penicillium genera, as well as yeasts: Rhodotorula, Candida and other pathogens (Listeria monocytogenes, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Proteus vulgaris, Salmonella). There are unique biological properties for isolated natural strains of Lactococcus lactis species. Most effective new bacteriocin-synthesizing strains 194 and K-205 were isolated from raw cow milk and kurunga from Buryatia. These strains have high antibiotic activity up 3600 and 2700 IU/ml compared to nisin and 2500-1700 IU/ml compared to fungicidal antibiotic nistatine. Treatment of the infected by Aspergillus repens raw smoked sausages with cultural broth of these strains of L. lactis subsp. lactis inhibited the spoilage. After treatment the sausages had prolonger shelf life and integrated products correspond to technological and microbiological indexes. The results of this study indicated that the treatment with lactococci strains can prevent a contamination of raw smoked sausages by potential food born pathogens. So, potential applications of bacteriocin synthesizing Lactococcus lactis subsp. lactis strains in various food fermentations, allow recommend its as potential perspective biopreservatives for preventing fungal spoilage of foodstuff and edible raw materials.

Keywords: antifungal bacteriocins, biopreservatives, lactococci, smoked sausages, spoilage.
Non-selective and selective isolation of DNA from food matrices and other real samples by magnetic particles

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Recent research has been focused on the development of innovative magnetic micrometer-sized particles for specific applications in DNA analysis in food matrices and other real samples. A rapid, small-scale DNA isolation method is needed to take full advantage of the speed potential of PCR technology. Non-selective and/or selective isolation of high-quality DNA can be performed using suitable functionalised magnetic particles. Carbonyl group-containing magnetic nonporous hydrophilic poly(2-hydroxyethyl methacrylate-co-glycidyl methacrylate) (PHEMA-co-GMA) microspheres were used for non-selective isolation of whole DNA from the sample. This procedure was used for PCR-ready DNA isolation and identification of Lachnocladium, Bifidobacterium and psychrotrophic bacteria in different dairy products or sediments. The magnetic microparticles P(HEMA-co-GMA) functionalised with streptavidin were used for immobilisation of biotinylated DNA probe and for selective isolation of target DNA from complex samples (e.g. food supplements) using DNA/DNA hybridisation. Isolation of target DNA was verified by PCR using genus-specific primers R16-1 and LBHMA-rev (Debarnet et al., 2002) in food supplements.

References

Keywords magnetic particles; streptavidin; DNA; PCR

The financial support of the Ministry of Education, Youth and Sports of the Czech Republic, grant No. 2B06053, and a long-term research programme MSM 0021622415 of the Ministry of Education of the Czech Republic are gratefully acknowledged.

Occurrence of spoilage bacteria *Pseudomonas* and *Pectobacterium* on Finnish carrots

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Carrot is the most important storage vegetable in Finland. Storage of carrot (*Daucus carota*) is a prerequisite for a year-round supply of domestic vegetables, but long storage period can impair the quality of carrot and storage diseases may cause considerable storage losses. Storage diseases are caused by pathogenic fungi, many species of bacteria and yeasts. Bacteria belonging to genera *Pectobacterium* and *Dickeya*, both formerly belonging to *Erwinia* genus, and *Pseudomonas* have been reported to cause soft rot on a wide variety of plants

Recent research has been focused on the development of innovative magnetic micrometer-sized particles for specific applications in DNA analysis in food matrices and other real samples. A rapid, small-scale DNA isolation method is needed to take full advantage of the speed potential of PCR technology. Non-selective and/or selective isolation of high-quality DNA can be performed using suitable functionalised magnetic particles. Carbonyl group-containing magnetic nonporous hydrophilic poly(2-hydroxyethyl methacrylate-co-glycidyl methacrylate) (PHEMA-co-GMA) microspheres were used for non-selective isolation of whole DNA from the sample. This procedure was used for PCR-ready DNA isolation and identification of Lachnocladium, Bifidobacterium and psychrotrophic bacteria in different dairy products or sediments. The magnetic microparticles P(HEMA-co-GMA) functionalised with streptavidin were used for immobilisation of biotinylated DNA probe and for selective isolation of target DNA from complex samples (e.g. food supplements) using DNA/DNA hybridisation. Isolation of target DNA was verified by PCR using genus-specific primers R16-1 and LBHMA-rev (Debarnet et al., 2002) in food supplements.

This study was undertaken to obtain knowledge on the bacterial pathogens on Finnish carrots by evaluating post-harvest spoilage of carrots during storage and marketing. The occurrence of *Pseudomonas* and *Pectobacterium* in carrots collected from farms and shops in Southern and Western Finland during two storage seasons was studied.

Genomic techniques adapted for the characterization of soft rot bacteria such as specific PCR, ARDRA (amplified ribosomal DNA restriction analysis), ITS-PCR-RFLP (intergenic transcribed spacer –PCR – restriction fragment length polymorphism) and RFLP-PFGE (pulsed field gel electrophoresis), were applied to identify the strains isolated from carrot samples and to characterize the genetic diversity of the isolates.

Using the methods described, 252 *Pseudomonas* isolates were identified from the carrot samples studied. According to the results of ARDRA with four selected restriction endonucleases, isolates as well as reference and type strains of *Pseudomonas* species could be classified into eight separate groups. Identification of carrot-spoiling isolates revealed that the majority of the *Pseudomonas* strains belonged to group I (*Ps. fluorescens*, *Ps. marginalis* and *Ps. veronii*) and group II (*Ps. putida*). High level of diversity within the *Pseudomonas* ARDRA group I (*Ps. fluorescens/Ps. marginalis/Ps. veronii*) and group II (*Ps. putida*) was revealed by genomic fingerprinting by RFLP-PFGE.

The reference strains of *Pe. carotovorum*, *Pe. atrosepticum* and *D. chrysanthemi* were clearly distinguished by ITS-PCR and digestion of the products with *Rsa*I. *Pe. carotovorum* was found in carrot batches studied. The samples infected by *Pectobacterium* were usually very soft and slimy. All isolates belonging to the genus *Pectobacterium* macerated carrot discs in pathogenicity tests. Heterogeneity was revealed on genotypic analysis of the type strains and isolates belonging to the genera *Pectobacterium* and *Dickeya*.

The three rDNA-based identification methods 16S ARDRA, 16S-23S rDNA ITS-RFLP and rDNA sequencing, supplemented with genomic fingerprinting by RFLP-PFGE allowed us to characterize the main spoilage bacteria involved in Finnish carrots and to explore the level of genetic differences of the isolates.

References

Keywords *Pseudomonas*, *Pectobacterium*, carrot spoilage; ARDRA; ITS-PCR; RFLP-PFGE
Ochratoxin A and ochratoxigenic fungi in coffee (Coffea arabica L.) in southern Minas Gerais State-Brazil (1998 to 2005)

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During the period of 1998 to 2005, 309 sample of beans coffee (Coffea arabica L.) from eleven producers locations have been analyzed at South of Minas Gerais State Region about the contamination by ochratoxigenic fungi and ochratoxin A. The levels of contamination varied from non detected to 181,66 μg/Kg in the different portion (portion of the product with particular characteristics) of coffee beans. The results have showed that among the analyzed samples, 78,11% have presented contamination levels between non detected to 5μg/Kg. The coffee of “varrição” and the coffee “boia” were theones that presented the higher levels of ochratoxin A, showing an advancedelevation in the prevention of the ochratoxin occurrence. It was also evaluated the fungi producers of ochratoxins A present in coffee beans. The main ochratoxigenic species are: Aspergillus ochraceus and A. sulphureus. Other producers species have also occurred, but with low incidence: A. sclerotiorum, A. petraki, A. elegans, A. ostiatus and A. auricomus. Considering only the contaminated samples and supposing there wasn’t any lost of ochratoxin A during the coffee roasting, the different samples would show different risks of exposition to ochratoxin A to Brazilian consumers. Considering the safe level of daily intake of ochratoxin A established by JECFA (Joint FAO/WHO Expert Committee on Food Additives), nearly 7200ng/day for an adult of 70 Kg weight, it would be a daily intake of 89μg/day; 81.75 ng/day and 36 ng/day less than 16.5 ng/day corresponding to 9.4%; 8.2% and 3.6%, of safe level allowed by JECFA for the consumption of coffee of “boia”, “varrição” and mix of cherry+green fruit, respectively.

Acknowledgements The support FAPEMIG and CNPq is gratefully acknowledged

Key words: mycotoxin, toxigenic fungi, Aspergillus, coffee

Optimization of honey-musts alcoholic fermentation to obtain high quality mead

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Mead is an alcoholic beverage containing 8-18% of ethanol, which results from the alcoholic fermentation of diluted honey performed by yeasts. The main problems encountered during mead production are slow or premature alcoholic fermentation arrest and the appearance of unpleasant odors in the final product. Therefore the aim of this work was to optimize mead fermentation process. For that purpose several fermentation conditions have been tested, based on oenological fermentations. Those manipulations included nitrogen supplementation, addition of malic acid and potassium tartrate as well as pH adjustment. A wine Saccharomyces cerevisiae strain known for its high fermentative ability was used in all assays. Yeast growth and fermentation performance was periodically monitored and at the end of alcoholic fermentation samples were collected for routine analysis, according to the official wine methods. Additionally, aroma compounds formation was also assessed by SPME coupled to GC-MS. The results obtained showed that nitrogen addition had a strong impact on yeast performance by reducing significantly the time required by yeast cells to complete alcoholic fermentation. In contrast, fermentative activity was not affected by the addition of acids and/or pH modification. While no significant differences were found on the final ethanol concentration, ranging within 10.7 to 11.4%, important quantitative variation on the volatile compounds were observed within treatments.

Further studies are under way in our laboratory to optimize this biotechnological process in order to obtained high quality mead.

This work was partially supported by FCT through the project PTDC/AGR-ALI/68284/2006

Keywords: Alcoholic fermentation; mead; nitrogen concentration, wine yeasts, Saccharomyces cerevisiae
Partial fermentation of must from Tempranillo dried grapes by selected yeasts

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Two batches of musts from dried grapes of the Tempranillo variety, with a sugar concentration around 500 g/L have been inoculated with two selected Saccharomyces cerevisiae yeast strains (X4 and X5) characterized by its osmoethanol tolerance. A third batch was fermented with the native yeast strains of the must. These batches were fermented to a final ethanol concentration of 9% (v/v) of ethanol. Fermentations were stopped by adding ethanol until 12% (v/v). A fourth batch of must was supplemented with ethanol to a final ethanol concentration of 12% (v/v) and used as control. Volatile aroma compounds were analyzed by gas chromatography and the results were statistically compared to establish differences among the batches. Significant differences among the fermented must and the control were observed in the carboxilics acids, lactones, esters and phenolics compounds. Odor activity values (OAV) of the volatile aroma compounds were calculated as the relation concentration-odor perception threshold. Grouping the OAV of the aroma compounds according its odor descriptor ten odorant series were obtained which can be used as a fingerprint of the resulting wines. Only the sweet series showed no significant differences among the four batches. Musts fermented with the selected yeast strains showed significant higher values in relation with the other two batches in the floral, fruity and toasty series. Lastly the organoleptic analysis showed that the must fermented with the X4 yeast strain was valued as the best in term of taste and aroma. This can be probably due to the metabolism of the yeast strain.

Key words: Aroma compounds; Dried grapes; Odorant series;
Polyphasic study of Lactococcus lactis isolates from diversified sources

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Lactococcus lactis is mainly used as a starter organism for milk fermentation. Lack of variability in the strains isolated primarily from the milk products is always a reason for screening of a new and improved strain as a replacement for the starter strains currently used in industrial fermentation. The present work aimed to explore the isolation of L. lactis from diversified sources. L. lactis strains have been isolated from dairy and non-dairy sources on the basis of their biochemical attributes. L. lactis sp. lactis and L. lactis sp. cremoris were further differentiated biochemically and confirmed genotypically, by using gadB gene sub-species specific PCR. Additionally, the strain typing was performed by (GTG)-REP PCR fingerprinting and diversity analysis was done by Ntys software package using simple matching coefficient and UPGMA clustering method.

A total of 139 isolates of L. lactis were identified from 45 samples collected from different regions of India. Out of 139 total isolates, 137 were genotypically identified as L. lactis sp. lactis (26 from dairy and 111 from non-dairy) while only two isolates (from yak milk and maize leaf) were identified as L. lactis sp. cremoris. A total of 12 isolates from plant, dahi, kefir and sprouted grains were found to be diacetyl flavor producing, thus characterized as L. lactis sp. Lactis bv. diacetylactis. It has been revealed that 9 isolates showing cremoritis phenotype were genotypically found to be lactis and 8 isolates did not show arginine hydrolysis, moreover one isolate genotypically confirmed as L. lactis sp. cremoris showed lactis phenotype. Interestingly, a total of 35 isolates of L. lactis sp. lactis from plants, sprouted grains and flowers were found to be able to grow under hostile conditions viz., 6.5% NaCl and pH 9.5. Thus, high levels of discrepancies have been found in phenotypic analysis. Therefore, identification of L. lactis subspecies was confirmed on the basis of the size of the product obtained after PCR amplification of gadB gene. L. lactis sp. lactis and L. lactis sp. lactis bv. diacetylactis generating 600bp band as compared to the 500bp band of L. lactis sp. cremoris. The isolates could be divided into 2 major clusters comprises of 39 sub-clusters of lactis and 2 sub-clusters of cremoris subspecies after REP PCR. Analysis of these sub-clusters revealed the presence of phenotypically different isolates in the same cluster and vice-versa. The simple matching coefficient between lactis and cremoris subspecies was found to be 78%. The percentage of genetic diversity of L. lactis sp. lactis, L. lactis sp. Lactis bv. diacetylactis and L. lactis sp. cremoris was found to be 90%, 9.6% and 1.4% respectively. As, cremoris subspecies has been rarely isolated form plants, but 1 strain of cremoris subspecies has been isolated from maize leaf in our study showed 96% similarity by 16s rDNA partial sequencing. Furthermore, to the best of our knowledge this is the first report for the isolation of L. lactis sp. lactis from flowers like Dianthus coronaria, Cassia fistula and Rosa cinnena as well as from sprouted grains like Phaseolus mungo, Vigna unguiculata and Cicer arietinum showing 99% to 100% similarity by 16s rDNA partial sequencing. Apart from these isolates, eight more L. lactis (seven of lactis and one of cremoris subspecies) strains isolated from vegetables, dairy products were partially sequenced and showed 98% to 100% similarity. It also has been demonstrated that after sequencing CL-sequences viewer software provided similar outcomes to the results generated by Ntys software package after REP-PCR. The nucleotide data generated were submitted to NCBI Gene Bank under following accession numbers are FJ668488, FJ660489, GQ267534, GQ267535, GQ267536, GQ267541, GQ267537, GQ267538, GQ267539, GQ267543 and GQ267542.

It has been concluded that, only phenotypic tests are unable to differentiate L. lactis subspecies, as phenotypic characters are the outcome of the interaction between genes and environment and thus can lead to the ambiguous results. Phenotypic results were not found to coincide with genotypic outcomes. REP-PCR was found to be reproducible and able to discriminate lactis and cremoritis subspecies but not its biovar diacetylactis. Plant-derived strains were found to be genetically close to the milk-derived greater strains but possess tolence to NaCl and arginine as compared to the milk-derived strains. Thus, isolation of some robust strains from non-dairy sources showed that these sources can be further explored to isolate and add new strains of lactococci to be used as dairy starters.

Keywords: L. lactis, isolation of L. lactis isolates, gadB gene specific PCR, (GTG)-Rep PCR

Preliminary selection of autochthonous Saccharomyces strains from Ronda (Malaga, Southern Spain) based on their oenological characteristics

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Wine fermentation is a complex microbial process involving the transformation of must into wine by the action of different species of yeasts and lactis acid bacteria originally present on the grapes and the winery equipment. The main responsible agents of the alcoholic fermentation are strains of the species S. cerevisiae. Commercially available dried yeast strains of S. cerevisiae can be inoculated into the grape juice in order to establish a high population and accomplish well-controlled must fermentations. However, the use of local, autochthonous strains of S. cerevisiae is preferable since they are better acclimatised to the environmental conditions thus assuring the maintenance of the typical sensory properties of the wines of a given region. Thus, for local strains selection purposes, it is necessary the isolation and identification of yeast species present in the fermenting must , together with their evaluation in accordance with established oenological criteria.

In a previous work, the yeast population present in a newly built winery from the Serrania de Ronda (D.O. Sierras de Malaga, Southern Spain) was studied. Yeasts were isolated from three different sources: i) Microvinifications (80 ml) carried out in laboratory using Merlot, Syrah and Cabernet Sauvignon grapes collected in the neighbouring vineyard, carried out during 2006 and 2007 vintages; ii) winery and equipment surfaces after 2006 vintage; and iii) grape musts at different stages of alcoholic fermentation from three vats (containing Merlot, Syrah and Cabernet Sauvignon must, respectively). Out of 1,582 isolates, 602 were identified as Saccharomyces cerevisiae by REP-PCR-RFLP of rDNA, and were further characterized by RFLP of mtDNA. This technique revealed a low level of polymorphism, with only 13 different mtDNA restriction patterns of autochthonous Saccharomyces (259 isolates) and 5 restriction patterns which matched to any one of the commercial yeasts employed since the first vinification in 2003 vintage. For further studies, the 5 commercial yeasts and the 8 autochthonous strains most frequently found, were selected. Their oenological properties and the aromatic characteristics of the wines obtained under laboratory controlled microvinifications were evaluated.

Firstly, some oenological properties such as low SH2 production, low foam production, and resistance to killer toxins were evaluated together with good enzymatic profile (high β-glucosidase and proteolytic activities). Other characteristics were also tested, such as the ability to ferment media with different glucose concentrations (225 and 275 g/L) at different temperatures (20 and 30 °C), exhaustion of sugar potential and high fermentation activity (> 0.2 g/L CO2), volatile acidity and ethanol production. No autochthonous or commercial strain satisfied all the first criteria. In fact, all but two of the autochthonous strains had β-glucosidase activity but only two of them showed proteolytic activity. Only one local strain did not produce SH2, but it produced foam. Almost all strains used sugar up to a level ≤ 5 g/L in both 225 g/L and 275 g/L glucose containing media at 20 °C, and in ≥ 225 g/L media at 30 °C, but none did it in 275 g/L media at 30 °C. Most of the strains produced ≤ 0.8 g/L of volatile acidity expressed as acetic acid in the 225 g/L glucose assays, but greater values were obtained in 275 g/L glucose fermentations. All strains produced at least 8.0 % vol alcohol in the 225 g/L sugar media, and all but one commercial and one local strain, at least 10.5 % vol in the 275 g/L sugar media. For further studies, two commercial and one local strain were discarded because they turned out to be sensitive to killer yeast toxins; the rest, were used for micro-scale fermentations (330 ml of sterile Merlot must) carried out under controlled conditions. The results showed that there were no great differences among them in the analytical parameters of the fermented products. However, sensory analysis results and volatile composition of the produced wines showed that autochthonous strains seem to produce wines with better quality. Finally, one commercial and four autochthonous strains were preselected for a pilot scale fermentation in the winery.

Keywords: Saccharomyces, oenological characteristics, selection

Acknowledgements: This study was supported by funds from the Andalucia Government (EXP 92162/11).
Presence of *Arcobacter* spp. contamination in fresh lettuces for human consumption

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Arcobacters are considered emerging foodborne pathogens causing diseases in domestic animals and diarrhoeal illness in humans. It has been suggested that water may play an important role in the transmission of these organisms. Raw meat is also considered as another source of *Arcobacter* infection in humans. In fact, *Arcobacter* spp. are frequently isolated from animal food products, in particular from poultry, as well as various types of water samples. However, there is no data on the presence of *Arcobacter* spp. in fresh vegetables and it could be interesting given that in recent years the consumption of salads has increased driven by the trend towards healthier eating. Therefore the objective of this research was to study the presence of *Arcobacter* spp. in fresh lettuces.

Fifty fresh lettuces purchased from different local shops in Valencia (Spain) between January and July of 2009 were analyzed. Because there is no standard method for *Arcobacter* spp. detection, the assay was performed simultaneously by PCR and cultural methods. The samples were analyzed directly and after 48 h enrichment in *Arcobacter* broth (AB) with Cephoperazone-Amphotericin-B-Teicoplanin (CAT) selective supplement at 30ºC under microaerophilic conditions. For PCR detection, 1mL aliquots from the broths were centrifuged at 12,000 rpm for 5 min, and DNA was extracted using a commercial food DNA extraction Kit. Then, an *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* species-specific multiplex PCR assay was performed according to Houf et al. (2000) with few modifications on temperature conditions (González et al., 2007). In addition, for isolation of bacteria, 90 µl of each broth was dropped on a 0.45 µm cellulose membrane filter which was placed on sheep blood agar plates with CAT. After one hour incubation at 30ºC in aerobic atmosphere, the filters were removed and the plates were incubated for 48 h at 30ºC under microaerophilic conditions. Presumptive *Arcobacter* colonies were selected from each plate, checked by Gram stain microscopic appearance, and subcultured onto blood agar plates. The cultures were purified and analyzed by multiplex PCR for species identification.

*Arcobacter* sp. was detected in 10 of the 50 samples (20%) by PCR, but just in one of them the detection was possible without enrichment. *A. butzleri* was the only detected species by multiplex PCR. Seven samples were found to be positive also by culture. Nineteen isolates were obtained, being all of them identified as *A. butzleri*. To our knowledge, this is the first report of *Arcobacter* spp. detection in fresh vegetables such as lettuces. Although these foods are generally considered safe and the incidence of major pathogens on raw vegetables is low, given the large quantities consumed and the fact that further cooking is absent, these foods could be a potential public health risk.

**Keywords** *Arcobacter* spp.; lettuces; culture; multiplex PCR

**References**


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Prevalence of *Salmonella* among foodhandlers in Owerri metropolis

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A study of *Salmonella* amongst food handlers in Owerri metropolis was carried out between February and August, 2006. This was done with a view to elucidating the level of hygiene employed by food handlers. Of the 50 stool samples collected 36(72.0%) had growth on the selective media used. After biochemical and morphological analysis. Because there is no standard method for *Arcobacter* spp. detection, the assay was performed simultaneously by PCR and cultural methods. The samples were analyzed directly and after 48 h enrichment in *Arcobacter* broth (AB) with Cephoperazone-Amphotericin-B-Teicoplanin (CAT) selective supplement at 30ºC under microaerophilic conditions. For PCR detection, 1mL aliquots from the broths were centrifuged at 12,000 rpm for 5 min, and DNA was extracted using a commercial food DNA extraction Kit. Then, an *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* species-specific multiplex PCR assay was performed according to Houf et al. (2000) with few modifications on temperature conditions (González et al., 2007). In addition, for isolation of bacteria, 90 µl of each broth was dropped on a 0.45 µm cellulose membrane filter which was placed on sheep blood agar plates with CAT. After one hour incubation at 30ºC in aerobic atmosphere, the filters were removed and the plates were incubated for 48 h at 30ºC under microaerophilic conditions. Presumptive *Arcobacter* colonies were selected from each plate, checked by Gram stain microscopic appearance, and subcultured onto blood agar plates. The cultures were purified and analyzed by multiplex PCR for species identification.

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**Keywords** Food handlers, Frequency, Morphological characterization, Infection, Isolates.

**References**


Prevention of food-transmitted human pathogen virus and bacteria in fruits and vegetables by use of indicator organisms

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Human pathogen viruses like Norovirus, which can be transmitted from one human to another is likely to be transmitted via foods and drinking water. Especially foods that are consumed without heating or other microorganism-reducing step are in the risk of being contaminated by human pathogen viruses and/or bacteria. Contamination can take place by use of contaminated water or due to inefficient hygiene of the labour.

Practically it is not possible to monitor virus contamination by traditional sampling because the methods available are very labour-intensive and hence too expensive to be part of the normal quality control procedures in the food industry. This work is done in order to offer the food industry an analysis tool to control the risk of foods to be contaminated by viruses. We have developed a real time PCR method that detects specific indicator organisms (Bacteroides dorei), that unambiguously links to contamination by human faeces/wastewater. The method can also be used to control process and drinking water quality.

The promising method as well as examples of its use in different contaminated fruits will be presented.

**Keywords** Indicator Bacteria, *Bacteroides dorei*, Food-borne viruses, qPCR, Faecal contamination,
Production of sweet probiotic milk using mixed culture of Lactobacillus and Bifidobacterium strains and studying its effect on IBD.

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A probiotic is a “live microbial food ingredients that, when ingested in sufficient quantities, exerts health benefits on the consumer. Probiotics exert their benefits through several mechanisms; they prevent colonization, cellular adhesion and invasion by pathogenic organisms, they have direct antimicrobial activity and they modulate the host immune response. We have many probiotic products. We try to product sweet probiotic milk by using mixed culture of indigenous lactobacillus and Bifidobacterium strains that are isolated in Iran (L.casei, L.plantarum, Bif.longum and Bif.bifidum). The milk has no fermented qualities, thus delivering the benefits without the high acidity and flavour, considered undesirable by some people.

These four strains were cultured in MRS broth, and then confirmed by biochemical tests. These strains were grown in Permeate media for 48 hours. The fresh sterilized milk(100 cc) was then inoculated with 5 cc 48 hours old pure culture of L.casei, L.plantarum, Bif.bifidum and Bif.longum and stored in 4 °C to reach sweet probiotic milk with concentration of 10^2 cfu/ml. The properties and permanency of this milk were investigated for pH ,taste, smell , number of probiotic microorganisms and its effect on treatment and prevention of IBD disease during 14 days at 4 °C, 25 °C, 37°C. In 4 °C, flavor and smell were constant for 14 days. After 12 days PH was about 5.80 (early PH was 6.6). Also total count demonstrated stability of bacteria and their growth during 14 days at three different temperatures. At 25 °C and 37°C after 14 days the PH reached to about 3. Flavour of milk became acidic after 4 days at 25°C and after 2 days at 37°C. Numbers of bacteria reach to 10^10 cfu/ml in a few days.

Studies in experimental animals give a clue about the potential application of lactobacilli and bifidobacteria to prevent or treat colitis, IBD.

The results confirmed it and showed a beneficial effect of probiotic bacteria in IBD. However, it must be kept in mind that data on the use of these agents in IBD are still very limited and not always consistent.

Keywords: Probiotic, Inflammatory Bowl Disease (IBD)

Pulsed light treatment for the shelf-life extension of packaged cooked ham slices

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Ready-to-eat (RTE) products are becoming very popular among consumer preferences in developed countries due to the current lifestyle and dietary habits. Some of these products are cooked meats which are prepared in small portions from heat treated blocks. Pasteurization ensures the inactivation of vegetative bacterial cells and provides a reasonable shelf-life to the product, but superficial recontamination during subsequent handling (i.e. slicing, packaging, etc.) may occur, which could affect both, preservation and safety. Among the new technologies that are being assayed for the higienization of RTE foods, pulsed light (PL) treatment seems to be adequate due to its efficacy for the decontamination of surfaces. The process consists of the application of short duration pulses (10^4-10^5 milliseconds) of an intense broad spectrum (200-1000 nm) light. The UV-C region of the spectrum (200-290 nm) is the main frequency band responsible for the bactericidal effect, which is primarily attributed to a photochemical damage on DNA (Wang et al., 2005). The present work is a study on the suitability of PL for extending shelf-life of packaged cooked ham slices.

Cooked ham blocks (10.5 cm diameter) were sliced and packaged in plastic bags (48 μm thickness polyamide/polyethylene/vinyl acetate–based copolymer) under aerobic and vacuum conditions. Preliminary studies were carried out in order to select the most appropriate fluence for PL treatment. In this way, microbial decontamination and sensory attributes (odour and visual appearance) were evaluated after applying 0.7, 2.1, 4.2 and 8.4 J/cm^2 (both sides of the slices were treated). The 8.4 J/cm^2 treatment was chosen for this study since it showed a good level of decontamination without modifying the sensory properties of the product. Control samples (non-pulsed) were also processed. Pulsed light treatment was applied in a desktop equipment Steribeam SBS-XeMatic-2L. Samples were stored at 4 °C and shelf-life was determined by periodical bacterial counting (TSA 32 °C, 24h) and sensory analysis (by a panel of 20 tasters).

The initial bacterial numbers in non-treated slices were approximately 2 log c.f.u./cm^2. However, in pulsed samples no counts were obtained immediately after PL treatment. Aerobically packaged untreated samples resulted sensorially unacceptable after 8 days of storage, although bacterial counts did not reach 6 log c.f.u./cm^2. In pulsed slices this condition was reached 4 days later. Vacuum packaging alone is a very useful tool to extend the shelf-life of sliced cooked ham. In control samples the lag phase was extended approximately one week and the product was acceptable until day 19 of storage. On day 26, a light hot culture medium odour appeared coinciding with counts of 5.7 log c.f.u./cm^2, followed by a clear vacuum packaged odour after 33 days of storage. On the other hand, in pulsed slices the lag phase lasted 26 days and samples were acceptable for 49 days, which means that PL treatment provided an additional shelf-life extension of 30 days in vacuum packaged slices when compared to non-pulsed samples.

This work was supported by the projects AGL2007-65235-C02-02 and CONSOLIDER-INGENIO 2010-CARNISENUSA (Ministerio de Educación y Ciencia, Government of Spain).

References

Keywords: pulsed light treatment, cooked ham, RTE foods, shelf-life.
Quantifying the effect of (in)organic acids on the thermal inactivation of *Escherichia coli*

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Introduction

The ability of stress adapted micro-organisms to resist when they are exposed to a different environmental stress is known as *cross protection* (Juneja and Novak 2003). It is widely known that acids and temperature are stress factors for bacterial cultures. The type of acid, used during acid stress may affect the level of stress and/or cell injury. Strong acids –such as HCl- lead to trafficking of the dissociated [H⁺] in the cell via the membrane, leading to a decrease of the internal pH of the cell to levels that can be toxic or lethal. The weak acids –such as acetic and lactic- enter bacterial cells in their undissociated form and they partly dissociate in the cytoplasm (Foster 2004). Weak acids are more stressful for the cells compared to a strong acid. The aim of this research is to investigate the influence of rapid pre-acid shock –with different types of acids each time- on the heat resistance of *E. coli* at lethal temperatures.

Materials and Methods

*E. coli* cells have been grown in Brain Heart Infusion broth until they reach the stationary phase (≈ 10⁹ cfu/mL). These stationary phase cells have been added in normal Brain Heart Infusion Broth (pH=7.4) and in pH re-adapted (pH in the range from 5 to 6) Brain Heart Infusion Broth. Inactivation experiments take place at constant, lethal temperatures for *E. coli*, and specifically at 54°C and 58°C. The duration of the pre-acid shock is approximately 30 minutes. The re-adaptation of the pH has been achieved with the addition of 50% (v/v) of different acids each time (acetic acid, lactic acid, hydrochloric acid). Induced resistance is defined as a prolongation of the shoulder and/or a reduction on the inactivation rate and/or an occurrence of a tail on the inactivation curve, indicating the presence of a stress resistant population.

Results

It is observed that rapid pre-acid shock can lead to resistance of *E. coli* to heat. The induced heat resistance is dependent on the type and the amount of acid used, since different levels of acidification (different pH values of the broth) lead to a different level of heat resistance. More specifically, addition of acetic acid in the medium for pH in the range of 5-6 always leads to an induction of resistance, the extend of which is dependent on the pH value for both studied temperatures. For addition of lactic acid in the range 5-6 a slight induction of resistance was observed at 58°C but no resistance was observed at 54°C. Addition of hydrochloric acid in the medium at 54°C leads to an induction of resistance which is similar for all the pH values in the range 5-6. At 58°C for pH 6 also a prolongation of the shoulder is observed and there is an occurrence of a tail. The presence of the tail is the result of a stress resistant population. The formation of this resistant population – which is not present in non acidified conditions - is possible that occurs due to the pre-acid shock, which increases the bacterial resistance to heat.

This work aims at providing additional quantitative knowledge on the reaction of high density bacterial cultures to acids and heat on the extent of their level of adaptation and induced resistance.

Acknowledgements

This work was supported by grant DR/08/006/BOF and KULeuven-BOF Projects OT/09/25 and EF/05/006 OPTIC: Optimization in Engineering of the Research Council of the Katholieke Universiteit Leuven, and the Belgian Program on Interuniversity Poles ofAttraction, initiated by the Belgian Federal Science Policy Office.

Keywords *E. coli*; pre-acid shock; heat resistance; acid stress; heat stress; predictive modeling

References

Quinolone resistance in nontyphoidal *Salmonella enterica*: role of chromosomal mutations and plasmid-mediated determinants

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The use of quinolones in antimicrobial chemotherapy is widening the spread of resistant microbial strains. Among those, *Salmonella* isolates are still rare, but they are becoming more frequent. The main mechanisms of quinolone resistance are mutations in the (chromosomal) quinolone resistance determinant region (QRDR) in the *gyrA*, *gyrB*, *parC* or *parE* genes. Moreover, resistance to quinolones might be acquired by conjugal mobilization of plasmids. The plasmid-mediated quinolone resistance (PMQR) originates a low level of resistance and is thought to enhance the selection of microorganisms with higher resistance level to quinolones by lowering the susceptibility threshold at which they can be selected.

This work focuses on the study of quinolone resistance in *Salmonella enterica* strains (n=364) isolated in humans from different locations in the mid-west of Spain (Cáceres), between the years 2004 to 2008. Minimum inhibitory concentrations for nalidixic acid, ciprofloxacin and enrofloxacin were determined by broth dilution, and the QRDR of *gyrA*, *gyrB*, *parC* and *parE* were sequenced. In addition, the presence of PMQR genes *qnrA*, *qnrB*, *qnrS*, *qnrC*, *qnrD*, *qnrF* and *qepA* were determined. According to the Eucast criteria, 34% of strains were resistant to at least one of the tested quinolones. From the resistant strains, 77% had a single *gyrA* mutation, 4% had a single *parC* mutation and 3% had both mutations. Strains with more than one mutation in *gyrA* or *parC* were not found. Resistant strains lacking mutations in *gyrA* or *parC* were screened for mutations in *gyrB* and *parE* genes, but no such mutations were found. The mutations in *gyrA* were often associated to serotypes Enteritidis and Typhimurium, while mutations in *parC* were exclusively found in serotypes different from the previously mentioned. A high-level of resistance to the quinolone nalidixic acid and a low-level of resistance to the fluoroquinolones enrofloxacin and ciprofloxacin were found associated with mutations in *gyrA*, *parC* or simultaneously with both genes in the same strains. The only PMQR determinants detected have been *qnrS* and *qnrB*, each one in a different strain of *S*. Typhimurium. Both strains lack mutations in the QRDR of *gyrA*, *gyrB*, *parE* and *parC* genes. The strain containing the *qnrB* determinant, carrying a new allelic polymorphism, presents high resistance to both quinolone and fluoroquinolones, whilst the strain with *qnrS* shows low-level resistance to nalidixic acid, enrofloxacin and ciprofloxacin. Thus, although chromosomal mutations (*gyrA* or *parC* coding sequences) or plasmid mediated (*qnrB* or *qnrS*) determinants can explain the majority of the strains' resistance phenotype, a fraction of them had resistance phenotypes relying on unknown resistance mechanisms.

**Keywords:** *Salmonella*, quinolones, QRDR, PMQR.

**Acknowledgements:** This work has been funded by grants AGL2006-04147 (Ministerio de Educación y Ciencia) and PR08B001 (Junta de Extremadura).

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Red Bacterial Cellulose Production by Fermentation of *Monascus purpureus*

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Red Pigment is one of the secondary metabolic products produced by *Monascus purpureus* strains. It has widely applications in many industries such as foods, pharmaceutics and cosmetics. Red pigment production were studied on bacterial cellulose derived from *Acetobacter xylinum* TISTR 975 by using *M. purpureus* TISTR 3002, *M. purpureus* TISTR 3385 grown in Modified Yeast Malt Extract supplemented with soybean flour.

The experiment was carried out on the rotating shaker at 250 rpm under 30 °C for 14 days. Result showed that rate of pigment production of 3385, 3002 and 3180 are 0.093, 0.036 and 0.029 UA, in that order. TISTR 3385 revealed the highest pigment production with the ratio of 1.18 and 1.68 times when compared with those of 3002 and 3180. The substrate consumption decreased as an increasing fermentation time. Result also showed that 3385 consumed substrate 1.22 and 1.85 times higher than 3180 and 3002 with the rate of substrate consumption 0.166, 0.138 and 0.091 g/L·day. Total color differences were also evaluated and showed that 3002 exhibited higher color yield than 3385 and 3180 about 2.25 and 3.78 times with the values of 1.51, 0.67 and 0.40 unit/day. Red bacterial cellulose concentrations for nalidixic acid, ciprofloxacin and enrofloxacin were determined by broth dilution, and the QRDR of *gyrA*, *gyrB*, *parC* and *parE* genes. Moreover, resistance to quinolones might be acquired by conjugal mobilization of plasmids. The plasmid mediated quinolone resistance (PMQR) originates a low level of resistance and is thought to enhance the selection of *Salmonella* strains with higher resistance level to quinolones by lowering the susceptibility threshold at which they can be selected.

This work focuses on the study of quinolone resistance in *Salmonella enterica* strains (n=364) isolated in humans from different locations in the mid-west of Spain (Cáceres), between the years 2004 to 2008. Minimum inhibitory concentrations for nalidixic acid, ciprofloxacin and enrofloxacin were determined by broth dilution, and the QRDR of *gyrA*, *gyrB*, *parC* and *parE* genes. In addition, the presence of PMQR genes *qnrA*, *qnrB*, *qnrS*, *qnrC*, *qnrD*, *qnrF* and *qepA* was determined. According to the Eucast criteria, 34% of strains were resistant to at least one of the tested quinolones. From the resistant strains, 77% had a single *gyrA* mutation, 4% had a single *parC* mutation and 3% had both mutations. Strains with more than one mutation in *gyrA* or *parC* were not found. Resistant strains lacking mutations in *gyrA* or *parC* were screened for mutations in *gyrB* and *parE* genes, but no such mutations were found. The mutations in *gyrA* were often associated to serotypes Enteritidis and Typhimurium, while mutations in *parC* were exclusively found in serotypes different from the previously mentioned. A high-level of resistance to the quinolone nalidixic acid and a low-level of resistance to the fluoroquinolones enrofloxacin and ciprofloxacin were found associated with mutations in *gyrA*, *parC* or simultaneously with both genes in the same strains. The only PMQR determinants detected have been *qnrS* and *qnrB*, each one in a different strain of *S*. Typhimurium. Both strains lack mutations in the QRDR of *gyrA*, *gyrB*, *parE* and *parC* genes. The strain containing the *qnrB* determinant, carrying a new allelic polymorphism, presents high resistance to both quinolone and fluoroquinolones, whilst the strain with *qnrS* shows low-level resistance to nalidixic acid, enrofloxacin and ciprofloxacin. Thus, although chromosomal mutations (*gyrA* or *parC* coding sequences) or plasmid mediated (*qnrB* or *qnrS*) determinants can explain the majority of the strains' resistance phenotype, a fraction of them had resistance phenotypes relying on unknown resistance mechanisms.

**Keywords:** *Monascus purpureus*, *Acetobacter xylinum*, bacterial cellulose
Risks of *Vibrio parahaemolyticus* in black tiger shrimps (*Penaeus monodon*)

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A study was undertaken to determine the risks posed by *Vibrio parahaemolyticus* in black tiger shrimps (*Penaeus monodon*) and culture environment. The prevalence of *V. parahaemolyticus* in frozen shrimps (30), live shrimps (40), sediments (38) and water (48) samples collected from a shrimp factory and 3 farms, respectively, were determined. A total of 251 isolates were tested including 60 from frozen shrimp, 50 from live shrimp, 67 from sediments and 74 from water targeting the toxR, thermostable direct hemolysin (*tdh*) and related hemolysin (*trh*) genes for confirmation of total and pathogenic *V. parahaemolyticus*. Microbiological risk assessment was conducted in order to estimate the risk of getting infected by consuming cooked shrimps for Malaysians and also for Japanese by consuming raw frozen shrimp imports from Malaysia.

*V. parahaemolyticus* was detected in 98% of water samples with densities ranging from 10 to 420 cfu/ml whereas it was 200 to 9000 cfu/g for pond sediments. *V. parahaemolyticus* was detected in all live shrimp samples with densities ranging from 300 to 8000 cfu/g. Frozen shrimp samples (43%) were positive for *V. parahaemolyticus* ranging from 4 to 93 MPN/g. The strains (51%) were found to be positive for toxR and 15% of the isolates from culture environment and 7% of the isolates frozen shrimp possessed the virulent genes. Estimated illness per year was 123 persons (age from 18 to 59 years) for Malaysian and 63 for Japanese people. This study indicated that pathogenic *V. parahaemolyticus* strains were present in shrimp culture environment in Malaysia and suggest a probable risk for health of people consuming raw shrimp.

**Keywords:** Microbiological risk assessment, *Vibrio parahaemolyticus*, black tiger shrimps

Screening of lactic acid bacteria from wine and grapes for malolactic and glycosidase activities

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Lactic acid bacteria (LAB) have significant impact on the composition and sensory expression of wine by conducting the malolactic fermentation (MLF). There are only a few species adapted to the harsh conditions in wine, mainly *Oenococcus oeni* and several species of *Pediococcus* and *Lactobacillus*. The main characteristic of MLF is the conversion of malic acid into lactic acid by the malolactic enzyme. Also the β-glycosidase activity of these species may play an important role in aroma enhancement of wine during MLF by the release of terpenic compounds from their glycosylated precursors.

Therefore, several methods have been developed to isolate LAB and to screen for malolactic and glycosidase activities. The sources of strains were malolactic starter cultures as well as samples from Austrian wines and grapes. The isolated strains were identified by microscopy, Gram staining, catalase tests and restriction analysis of the amplified 16S rRNA gene.

To screen for malolactic active strains a selective medium containing bromocresol green to indicate deacidification was developed, HPLC analysis was used to verify malate decarboxylation.

Screening for glycosidase active cells was made by streaking onto agar minimum media plates with 4-methylumbelliferyl-β-D-glucopyranoside (4-MUG) and 5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside. Furthermore, the glycosidase activity of the isolated strains was determined by their ability to hydrolyze p-nitrophenyl-β-D-glucopyranoside.

**Keywords:** lactic acid bacteria, malolactic fermentation, glycosidases
Selection of a broad lytic spectrum phage for Salmonella detection
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Salmonella infection continues to be a major cause for food-borne illness throughout the world. Therefore, the rapid detection and identification of this pathogen is extremely important to maintain public health safety. Many conventional methods currently used take several days, therefore new and expeditious methods are being developed, based on the direct interaction of the pathogen with a specific bioelement, such as antibodies, DNA, enzymes and very recently bacteriophages. Phages are viruses that detect and eliminate specific bacteria. As such, they have been applied in phage therapy, water treatment, high-throughput screening and also biosensing. Moreover, compared with other bioelements, phages are more stable and less sensitive to environmental stress, such as pH and temperature fluctuations. Also, their production costs are very low.

In order to obtain a phage suitable to serve as a bioelement, we analysed the lytic spectrum of several Salmonella phages (isolated in the scope of the European project Phagevet-P) against Salmonella sp. and E. coli, among other bacteria. The results showed that Salmonella phage phi PVP-SE1 had the broadest lytic spectrum among all tested phages. The morphology of phage phi PVP-SE1 was analysed using TEM. It was shown to have a contractile tail and resembles typical 01-like phages that belong to the Myoviridae family. Comparing the lytic spectrum of this phage to the well known Felix 01 (a virulent phage originally isolated by Felix and Callow, on the different isolates, we observed that phi PVP-SE1 presents a broader host range than Felix 01. Felix 01 only lysed 75% of de strains lysed by phage phi PVP-SE1. This is interesting as Felix 01 is routinely used as a diagnostic tool in the identification of Salmonella due to its capability to lyse up to 99.5% of Salmonella strains. The broad range lytic spectrum makes the phi PVP-SE1 a potential tool in phage therapy, because it may cover many types of Salmonella strains, which is a huge advantage as method of detection.

Despite having a high throughput, conventional microbiological detection techniques such as enzyme linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) are time consuming, and require expertise and suitable laboratory conditions. Therefore, this newly isolated phage phi PVP-SE1 can be an excellent element of choice to include in the construction of an advanced and user-friendly biosensing system with high levels of specificity, selectivity and stability.

Keywords: Bacteriophages; Salmonella sp.; Felix 01; lytic spectrum; Biosensing system

Selection of lactic acid bacteria for the production of phenyllactic acid for food conservation
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The growing interest of consumers towards the use of high quality foods that are more natural and minimally processed, while being safe and with a long life, together with stricter legislation regarding the preservatives used, has challenged for the food industry and has led to increased research and use of natural preservatives. Today it is known that certain lactic acid bacteria (LAB) produce antifungal, thus offering a potential alternative to the use of synthetic preservatives.

These LAB have many applications in fermented food production as a major influence on nutritional, sensory and shelf life of products. LAB are known to produce bioactive molecules (such as organic acids, fatty acids, hydrogen peroxide, diacetyl and bacteriocins) showing antimicrobial activity against organisms and pathogens. Due to their antifungal activity, Lactobacillus plantarum has been investigated especially in connection with the production of organic acids and/or cyclic dipeptides, showing for example, that L. plantarum ITM21B, used as initiator in yeast bread, slows the growth of Aspergillus niger and Penicillium roqueforti for 7 days and significantly prolongs the life of bread (1). In the filtrate of the culture was observed the presence of phenyllactic acid (PLA) and its derivative 4-hydroxy phenyllactic (OH-PLA). There is evidence that PLA is a novel antimicrobial compound, first found in Geotrichum candidum and found to inhibit growth of Listeria monocytogenes (2). It is also active against Gram-positive and Gram-negative bacteria and fungi (3). It has also been observed inhibitory properties of PLA against several species of fungi isolated from bakery products, flour and cereals, including species such as Aspergillus ochraceus, Penicillium verrucosum and P. citrinum, and certain contaminating bacteria such as Listeria spp., Staphylococcus aureus and Enterococcus faecalis (4).

It has also been reported that PLA and OH-PLA are metabolites produced by LAB strains through the degradation of phenylalanine (Phe) and tyrosine (Tyr). Therefore, this work evaluates five strains of LAB (L. plantarum, L. acidophilus, L. pentosus, L. rhamnosus and Lactococcus lactis) to assess their ability to produce PLA and OH-PLA. Another aim is to investigate the influence of the metabolism of Phe in the formation of PLA in these strains. Consequently, different carbon sources (syntetic or with an agroindustrial waste origin) as well as different nutrient sources, all of them with different Phe content, were evaluated.

Keywords: phenyllactic acid, phenylalanine, lactic acid bacteria

Selection of \textit{Oenococcus oeni} strains to employ as starters in malolactic fermentation.


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Malolactic fermentation (MLF) is the second fermentation of wine that causes significant changes of wine sensory properties: in fact the acidity of wine reduces and the organoleptic characteristics of the final product improve. This fermentation is principally carried out by \textit{Oenococcus oeni}. Sometimes its performance has some problems and the use of commercial starter often does not help to enhance its management.

A selection of \textit{O. oeni} strains was made in the collection of the CRA- Centro di Ricerca per l’Enologia to test their speed of adaptation in wine and the speed with which they complete MLF.

Screening was made with different steps evaluating the growth rate of each strains: in the first step the growth at 20\textdegree{} C in MRS broth within 7 days was tested; in the next steps different media with stressing conditions were used: poor sugar and with the addition of compounds that make them similar to the wine such as malic acid (3g / L) and ethanol (10%) the last selection step was done in Nebbiolo wine. The evolution of lactic acid was monitored by HPLC.

Macrorestriction analysis with Sfi I and Apa I endonucleases and subsequent PFGE was carried out in order to identify the strains obtained at the end of the selection. Moreover MLST (Multilocus Sequence Type) analysis was applied by sequencing recP, gyrB and ddl genes.

Then the selected strains have been used as starters in Nebbiolo wine both individually and in pairs; a control trial was made by inoculating a commercial starter widely employed in winemaking. The process of MLF was monitored by HPLC and the sensory analysis of the obtained wines was made.

The results showed that the starters selected in this study were able to complete MLF in wine. In particular, the strains used in mixture completed MLF more rapidly than the commercial strain. Moreover after sensory analysis, performed with a trained panel, it was proved that the mixture allowed to obtain a wine-quality comparable with the one obtained using the commercial strain.

\textbf{Keywords} \textit{Oenococcus oeni}, Malolactic fermentation (MLF), starter selection, molecular characterization, sensory analysis

Spatial distribution of bacterial colonies in a model cheese

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In cheese matrices whatever the cheese variety, bacteria are immobilised and grow as colonies. Bacterial colonies are the bioreactors of ripening processes. Therefore, their distribution as well as the distance between them is of major importance for ripening steps since metabolites (carbon sources and bacterial products) must diffuse within the cheese matrix. It has been previously shown that the distance between colonies as well as their diameter could influence pH microgradients between colonies in gelatine (Malakar \textit{et al.}, 2000). No data is available up to date about spatial distribution of bacterial colonies and distances between them within the cheese. Our purpose was to explore this distribution and which factors it may be influenced by (quorum-sensing, inoculation rate...).

We had two approaches: (1) experimental measurements were obtained by using a GFP-\textit{Lactococcus} strain in inoculated UF (Ultrafiltration) cheese and taking stacks of confocal microscopy photos of the fluorescent colonies at different inoculation rates in order to check if colonies were randomly distributed and if they all developed in cheese. Theoretical 3D distances between colonies were performed on the basis of a random distribution and the development of all the colonies.

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Numerations showed that the final numbers of cfu/g was identical whatever the inoculation rate. By the first approach, we confirmed that bacterial colonies in the UF model cheese were effectively randomly distributed, accurately fitting Poisson's model. We demonstrated that the initial inoculation rate strongly influenced the distances between colonies, but also their diameter. Theoretical 3D distances between colonies (from 55 \textmu{}m at inoculum 10\textsuperscript{6} cfu/mL to 257 \textmu{}m at inoculum 10\textsuperscript{7} cfu/mL) were calculated added with the diameters of colonies. In order to experimentally validate these theoretical values, a mathematical model is under construction to calculate experimental 3D distances between colonies and their diameters in the cheese matrix. For this model, we have to face two major problems: the elliptic distortion of colonies by the confocal microscopy, and the attenuation of fluorescence in depth within the cheese matrix which have to be corrected.

It is the first study to demonstrate that bacterial colonies are randomly distributed when growing in a cheese matrix and to calculate distances between bacterial colonies in cheese according to the initial inoculation rate. These new results are crucial in the understanding of the mechanisms of ripening at a microscopic scale. The technological implications of this varied spatial distribution will be discussed.

\textbf{Key words} bacterial colonies – cheese – lactic acid bacteria – spatial distribution – modelisation – confocal microscopy
Species specific PCR detection protocol for the main mycotoxin-producing *Aspergillus* species in paprika.

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Paprika is an important food additive in many countries. Extensive data recently available indicate mycotoxin contamination, being *Aspergillus* the genus most frequently isolated from the paprika samples analysed. The rapid, specific and sensitive PCR methods represent a useful strategy to predict the risk of the most important mycotoxins and to assist strategies to prevent them entering the food chain. In this work, we have developed a protocol to detect the main aflatoxin (*A. flavus* and *A. parasiticus*) and ochratoxin A producers (*A. niger, A. carbonarius, A. ochraceus, A. westerdijkiae* and *A. steynii*) by specific PCR assays in paprika. We have evaluated several rapid commercial DNA extraction kits and determined the appropriate sample size for detection of the critical toxigenic species (visualization of a specific PCR product on agarose gel).

We have checked 23 paprika samples testing different sample size for DNA extraction with commercial kits (0.1 and 1 g). Subsequently, samples were incubated in Sabouraud-Chloramphenicol broth to obtain three different time points (0, 1 and 2 days). Finally, DNA was extracted with several commercial kits and tested for specific PCR assays previously developed in our group.

Incubation of the samples increased the percentage of contaminated samples (from 16 % at time 0 to 46% after 2 days of incubation). The highest number of species detected was also observed after two days of incubation (when all the species tested could be detected), being *A. flavus* the predominant species.

In conclusion, the paprika samples had high contamination levels and the optimum results were obtained starting from 0.1 g of paprika, incubating the samples for 2 days and using the DNeasy Plant Mini Kit extraction kit.

Keywords Paprika; Mycotoxins, *Aspergillus*

ß-lactam resistance and extended-spectrum ß-lactamases in *Salmonella* strains isolated from animals.

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Human salmonellosis is recognized as the second most prevalent food-borne disease in the European Union. Although the disease is normally self-limiting, it requires antimicrobial chemotherapy for children, elderly and immunocompromised patients. A mortality of 0.1% has been reported associated to salmonellosis in the European Union, mainly produced by strains expressing extended-spectrum ß-lactamases (ESBL) or quinolone resistance.

A collection consisting of 203 non-typhoid *Salmonella* strains (27 different serotypes) isolated from slaughtered animals or clinical cases by the Infectious Diseases Units of three veterinarian faculties (Cordoba, Cáceres and Madrid) between 1998 and 2008. Minimum inhibitory concentrations (MICs) for cefalotin, cefotaxime, cefquinome and cefazolin were determined by microdilution broth and 56 resistant strains were found. The presence of *bla*OXA-1, *bla*TEM, *bla*SHV, *bla*CMY and *bla*CTX-M genes was analyzed among the resistant strains, and the ß-lactamase activity was calculated in all strains containing resistance determinants. The *bla*TEM gene was detected in 13 strains, belonging to 7 different serotypes, whilst *bla*OXA-1 gene was found in 13 strains of *S. Typhimurium*. In general, *bla*TEM positive strains were more resistant to cefalotin (1st generation cephalosporine), and more sensitive to cefquinome (4th generation cephalosporine) than *bla*OXA-1 positive strains, although no significant differences were observed with 3rd generation cephalosporines. The ß-lactamase activity expression was higher for *bla*TEM than *bla*OXA-1 genotypes.

Keywords: *Salmonella*, resistance, ß-lactamases

Acknowledgements: This work has been funded by grants AGL2008-04147 (Ministerio de Educación y Ciencia) and PR08B001 (Junta de Extremadura).
Study of cell envelope proteinase systems of natural isolated thermophilic lactobacilli


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Clinical ecology data show that 84% of world population suffers from food allergies, and particularly in 60% cases, the reason of such allergies is milk. As far as cow milk is a source of proteins, essential amino acids, fats and carbohydrates, its exclusion from diet is undesirable. On the other side the high allergenicity of milk makes us to seek new ways for the solution of this problem. The most appropriate way is the use of LAB, which pre-digeste milk proteins, especially casein.

Otherwise, k-protein bioactive peptides derived from milk proteins are inactive within the sequence of the parent protein and can be released and activated only by proteolysis which can be accomplished by the action of proteinases and peptidases from LAB. The proteolytic system of LAB consists of cell wall bound proteinases and several intracellular peptidases. Transport systems specific for amino acids, di- and tripeptides and oligopeptides (up to 18 amino acids in length) are present in LAB and serve for the nitrogen uptake. Longer oligopeptides, not transported into the cells, can be a source for the liberation of bioactive peptides in fermented milk products when further degraded, for example by intracellular peptidases of LAB after cell lysis. In the human gastrointestinal tract, digestive enzymes will contribute to the further breakdown of long casein-derived oligopeptides, which may also lead to the release of bioactive peptides. Once liberated in the body, bioactive peptides may act as regulatory substances.

Objects of research were more then 30 LAB strains isolated from matsun and cheese samples collected from small farms in different regions of Armenia. All of them were screened on proteolytic activity, and only two strains exhibited proteolytic activities - L. bulgaricus and L. salivarius. These two strains were identified on their morphological, cultural, biochemical and physiological properties. These strains were analyzed by two methods for casein hydrolysis activities: skim milk (1) and milk-citrate (2) systems. Both total casein and β-casein were used as substrates in all experiments. However, none of these strains hydrolyzed whey proteins. To determine the optimal pH for casein hydrolysis, whole cells were incubated at various pH values using both Na-caseinate and β-casein as substrates. At 37°C the optimal pH was 6.6 for both strains. In parallel, temperature optimum (measured at pH 6.6) for each strain was 45°C. Finally, determination of caseinolytic activities was carried out at optimal pH and temperature for each strain and these samples were used for analyses by HPLC. Comparative study of hydrolysates of these strains revealed differences in hydrolysis profiles. The study of the influence of inhibitors on the proteolytic activity of whole cells revealed that mercaptoethanol, ethanol and iodoacetic acid had no effect. In the presence of a chelating agent such as EDTA (inhibitor of metalloproteinases), the activity of proteinases was reduced to some extent. PMSF (serine proteinase inhibitor) inhibited proteinases of L. bulgaricus and L. salivarius too. It can be concluded that both strains produce serine-type proteinases.

So the cell-surface proteinases of L. bulgaricus and L. salivarius may represent a novel source of proteinases. Mentioned LAB can be used for starter culture construction not only in production of cheese and matsun, but in production of new probiotics.

Keywords: lactic acid bacteria, proteolytic activity, pH-temperature optimum of hydrolysis, type of proteinase

This work performed at supporting of NATO SfP 982 164 grant and by PC “Vitamix-E”.

Sublethal injury, growth and inactivation rates of stressed E. coli O157:H7

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This study determined the responses of E. coli O157:H7 to resuscitative challenge in nutrient medium and inactivation challenges in unheated and heated apple juice following prior exposures to mildly and moderately injurious combinations of pH (3.0-8.0), osmotic pressure (αw=0.93-0.99) and temperature (3.0-62 °C) stresses. The individual and interactive effects of the stress factors on the measured E. coli responses were determined by multiple regression analyses.

Results showed that sublethal injury was significantly influenced by the individual linear and curvilinear effect of prior exposures to αw and the linear effect of temperature stress. On the other hand, growth lag times of the organism was influenced by the individual effect of pH and the pH-temperature, and αw-temperature interactions. On the contrary, the microbial growth rate and maximum population was not influenced by any of the stress factors. Acid inactivation rates, measured in decimal reduction times, in unheated (25 °C) apple juice were significantly influenced by the individual effect of αw and the interactive effects of pH-temperature and αw-temperature. Moreover, the decimal reduction times of E. coli O157:H7 in heated apple juice (55 °C) was influenced significantly by the individual effects of prior pH and αw stresses.

By correlating the % sublethal injury and lag times of the organism, results showed that injury rates alone may not correctly predict the subsequent resuscitation of the organism. The nature of the resulting injury contributed by specific stress factors should be considered. Occurrence of injury did not induce resistance to subsequent acid challenge. On the other hand, sublethal stress due to low pH was found to result in enhanced thermal resistance of E. coli O157:H7. The results obtained in the study contribute to futher understanding of how the test pathogen respond to environmental stresses commonly encountered in food and food processing ecologies; and how such responses are related to cellular stress and damage. These results may have significant implications in the improvement of food safety evaluation and control.

Keywords: acid inactivation; acid stress; E. coli O157:H7; heat inactivation; osmotic stress; sublethal injury; temperature stress
Sugar Cane Waste as Alternative Medium for Astaxanthin Production by Mucor javanicus

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Astaxanthin is the carotenoid widespread commercially, and has high economic value, primarily in the cosmetic, food and feed in order to pigmentation of animals such as poultry and fish, especially trout (Trutta trutta, Trutta fario) and salmon (Salmo salar). Astaxanthin is considered an important and powerful antioxidant, many times greater than other carotenoids, mainly due to its activity as a precursor to vitamin A, a substance effective in fighting various diseases. Astaxanthin can be obtained from natural sources or synthetic. The chemical synthesis can result in carotenoids denatured, reduced efficiency and, therefore, there is a preference in using astaxanthin from natural sources, extracted from some shellfish or obtained by microbiological process. These investigation looking for the accumulation of astaxanthin in the cells of filamentous fungi Mucor javanicus. The effects of different sugar cane media, including sugar cane juice and molasses, and the standard medium was used as control of carotene production. The illumination of blue lights Emitting Diodes (LEDs). The influence of the time inoculum padmization (107 spores/mL), pH 6.5, and 96h, 150 rpm, 25ºC were evaluated. The system of solvent for the astaxanthin extraction was acetone. The carotene determination was carried out using spectrophotometer at 470 wave length. In the presence of blue LEDs using the sugar cane juice, 4%, the concentration of astaxanthin was triplicate (without light, 3.4 μg/g of biomass, and with light, 10,2 μg/g of biomass). With molasses (7% and 10%) the light influence in the concentration of astaxanthin, but the concentration was almost the same (without light, 3.8 μg/g, and 3.5 μg/g, respectively and with light, 2.4 μg/g, and 2.8 μg/g, respectively).

Keywords: sugar cane juice, carotenoids, agro-industrial waste, filamentous fungi, astaxanthin

Supercritical fluids for pasteurization - on-line investigation of the inactivation mechanisms

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Fungi, moulds, bacteria and viruses represent a wide range of possible threats for bioprocesses on one hand and for the human body on the other. Conventional processes for inactivation of microorganisms using heat-treatment or gamma-irradiation do not meet basic requirements concerning consumer demands and cannot preserve nutritional or physico-chemical properties of the product. Thus, the strive for alternative inactivation methods has been going on for decades [1].

High-pressure CO2 (HP-CO2) can be used in an innovative and environmentally friendly process. The method has shown to be applicable for food pasteurization without causing organoleptic alterations [2, 3]. Recent investigations have clearly proven that besides pathogenic vegetative bacteria also spores can successfully be inactivated using compressed fluids with additional dissolution of oxidizing agents. Thus the process seems to be also suitable for sterilization of e.g. thermolabile pharmaceuticals or implants [4, 5].

However, the inactivation mechanisms (cell perforation, enzyme denaturation etc.) using highly compressed fluids, had not been fully understood yet [6]. The present study investigates a pH drop of non-buffered medium (extra-cellular) during HP-CO2 treatment. So far speculative intracellular mechanisms that are responsible for the inactivation, particularly pH drop, have been determined in the present work for the first time on-line at pressures up to 90 bar. The newly developed measurement system, however, allows measurements even at much higher pressures. The pH within the cytoplasm of various organisms - including acid tolerant Listeria species - has been investigated using fluorescent indicators: cFDA-SE and cFDCA-SE (covering pH values between 6.5 and 3.0). The results of this work also strongly support the hypothesis that the inactivation mechanism using compressed CO2 is based upon the acidification of intra-cellular liquids of treated cells and not of the extra-cellular medium. However, pressure induced permeabilization of the membrane seems to be induced by non-acidifying fluids as well.

The presentation will show that the eco-friendly treatment is a genuine alternative to conventional disinfection and sterilization processes and could boost innovative developments not only within the field of food treatment but also in medicine, drug processing and biotechnology.

Survey, identification and control of aflatoxigenic fungi and aflatoxins in grains and nuts.

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The fungal presence is associated not only the question of deterioration of grain or derivatives, but mainly to the contamination of food by toxins produced by certain fungi, called mycotoxins. Among the mycotoxins found in food sources, aflatoxins are the most important. They are products of secondary metabolism of fungi and Aspergillus flavus and Aspergillus parasiticus are the major producers. They are a group of at least 16 derivatives bisfuranoisocumarínicos, and the four main naturally produced aflatoxins are B1, B2, G1 and G2. Characterized by show carcinogenic, mutagenic and teratogenic effects, the problem of the presence of these toxins in food becomes even more serious because the processes for their inactivation in foods are not effective. In addition, the eliminating of only small parts of such substances can cause undesirable changes in foods, such as loss of nutrients and changes in aroma and flavor of products. In Brazil, the occurrence of aflatoxins has been observed frequently, and at high levels in foods used for human consumption and animal feed such as corn, peanuts and dairy. The contamination of peanut products as mentioned products and other sweets, took prominent public health relevance, given that children are the main consumers of these products. Added to this scenario, the production of feed, produced mostly by cereals that are not as well monitored when compared to studies for mycotoxins in food for human consumption. It stands out, thus a global concern for the high probability of contamination by the toxin of these animals and the possibility of transmission of toxic mycotoxins to meat, milk and eggs, resulting in a potent risk to human health. In this sense, a scientific research is being conducted by a team from the Federal University of Lavras - UFLA and the Institute of Agricultural Research of Minas Gerais - IPAMIG, in order to obtain a natural preservative able to control the development of toxicogenic fungi in grains. The activities that comprise this research began the construction of a fungal germplasm consisting, until this moment, of toxicogenic species of Aspergillus flavus and Aspergillus parasiticus isolated from various grains (peanuts, corn, coffee, nut, Pará), consisting of 43 isolates previously identified and cataloged. Tests in vitro of essential oils anise (Pimpinella anisum L.), ginger (Zingiber officinale R.), peppermint (Mentha piperita L.), sage (Salvia officinalis L.) and thyme (Thymus vulgaris L.) on mycelial growth and sporulation of Aspergillus flavus and Aspergillus parasiticus and production of aflatoxins indicated their potential antifungal and inhibition synthesis of aflatoxins. The encapsulation of essential oils with inhibitory effects observed in vitro step for tests in vivo have also shown promising results. Finally, a proposal is being discussed with researchers from the University UMinho, Braga / Portugal, for the implementation of proteomic analysis of different isolates of the species Aspergillus flavus and Aspergillus parasiticus isolated from different grains and nuts grown in Brazil. The aim is to verify different expressions of aflatoxin production among different isolates and to compare the fungal production with fungal isolates obtained from grain grown in Portugal.

New antibacterial molecules produced by endophytic Paenibacillus polymyxa

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Inoculation of commercial yeasts in wine-making process has become a common practice in most wine-producing regions. Active dry yeasts (LSA) have been selected on basis of their oenological properties and, therefore, they guarantee a successful vinification process increasing fermentation speed and reproducibility of wine characteristics. Since wine quality is significantly affected by the strain of Saccharomyces cerevisiae leading fermentation, the use of LSA can contribute to loose some typical sensorial properties of wines. In addition, the introduction of LSA may affect the diversity of indigenous population of yeasts in the winery, and they are even able to become the dominant strains in spontaneous fermentations.

The aim of this work was to study the incidence of commercial yeasts on the native S. cerevisiae strains diversity and their ability to lead spontaneous fermentations in the experimental winery of Estación de Viticultura e Enoloxía de Galicia (EVEGA). To do this, several spontaneous fermentations were carried out in the experimental cellar of EVEGA for a period of 7 years (2002 to 2008). Samples were taken from must and at the beginning, vigorous and final fermentation. A representative number of colonies was isolated from each sample. S. cerevisiae strains were characterized by analysis of mitochondrial DNA restriction patterns (Querol et al. 1992, System. Appl. Microbiol. 15: 439-446).

The results showed that ten different strains of S. cerevisiae were found as dominant strain or in codominance with other strains from a total of 64 spontaneous fermentation processes studied. Almost all of them showed genetic profiles (mtDNA-RFLPs) similar to those found in different commercial yeast that had been previously used in EVEGA cellar. Although more than 20 different strains of autochthonous S. cerevisiae strains were identified from these fermentations, only a reduced number of them were able to reach implantation frequencies as high as commercial yeasts did.

These results clearly indicated that commercial wine yeasts are perfectly adapted to wine cellar conditions and they successfully compete with the indigenous strains of S. cerevisiae even during spontaneous fermentations. On the other hand, autochthonous dominant strains that presented desirable oenological traits could be of interest to preserve wine typicality.

Keywords: spontaneous fermentations; commercial yeasts; S. cerevisiae strains
Synergism of natural compounds in struggle for safe and healthier food

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Nowadays consumers expect safe food products with excellent taste and appearance, produced without any use of chemicals. In order to fulfil the commission food producers apply modern processing techniques and use preservative additives. Physical preservation supposes to rapidly inactivate undesired bacteria. The role of different means of packaging is to sustain sterile conditions, while preservative supplements suppose to guard the safety of the food product by suppressing the uncontrolled growth of pathogenic and spoilage bacteria. Unfortunately many additives influence the sensory characteristics as colour, texture, flavour or taste of the foodstuffs, what results in low consumers’ acceptance. Additionally, strategies used to reduce spoilage and pathogenic bacteria are not selective enough and may inactivate also desired microflora. Food is usually overdosed with antimicrobials which are supplemented ‘in case’. Little is said about impact of such ‘bacteriostatic food’ on human microflora and human health. Unfortunately consumer of XXI century is condemned to buy unhealthy food in one place and ‘health’ in another under the form of pro-biotic bacterial cultures, served in plastic cups or other healthy food supplements. That’s why food producers must reach for natural preservation methods truly harmless to humans.

Nature offers wide spectrum of biologically active (phyto)chemicals which may be used as potential natural preservatives. Many characterise desired taste and, therefore have been used in cuisine for hundreds of years as flavourings and spices. Antibacterial compounds are detected in all parts of plant: leaves, flowers and their petals, fruits, stems, seeds, roots etc. These are mostly acids, alcohols, medium and long-chain organic acids (especially lauric acid, caprylic and capric acid), terpenic compounds and their derivatives. Probably their organic lipophilic character improves their affinity to the components of bacterial cell membrane, facilitates their penetration of the cell, and possibly improves transport of other bacteriostatic compounds to the cell, what increases their bacteriostatic properties. Plant extracts are effective not only against bacteria but also yeasts, fungi and viruses; for example water as well as alcoholic plant extracts containing alkaloids show antiviral effects against hepatitis B virus and HIV.

The effectiveness of plant extracts e.g. synergism between terpenoids and medium chain fatty acids was studied in liquid medium, simulating environment of cured cooked meat. Tested bacterial strains constitute typical spoilage microflora in vacuum (Lactobacillus curvatus) and MA-packed (Brochothrix thermosphacta) meats. Results were also validated in meat environment.

Lactobacillus curvatus was observed to be very resistant against used either terpenoids or fatty acids alone, while its growth was strongly inhibited when both types of products were applied together. Growth of Brochothrix thermosphacta was significantly inhibited when antimicrobial compounds were applied alone, while blend of terpenoids and fatty acids showed to be bactericidal.

keywords: food preservation, functional food, synergism, plant extracts.

The prevalence of Vibrio parahaemolyticus in sea foods in Isfahan, Iran

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Background: Vibrio parahaemolyticus is widely distributed in marine environments and is associated with gastroenteritis cases caused by consumption of contaminated seafood. Clinical manifestations of V. parahaemolyticus infections include diarrhea, abdominal cramps, nausea and vomiting. V. parahaemolyticus food poisoning was mainly caused by consumption of seafood such as fish, crab, shrimp, shellfish and mollusks. However, other food products may also be cross-contaminated. The objective of present study was to investigate the vibrio species contamination in various type of fish and shrimp in Isfahan.

Methods: A total of 411 fish and shrimp samples were collected from 18 fish stores in Isfahan. The samples were obtained between 2003-2004. The prevalence of vibrio species was determined by using pre-enrichment in selective media and streaking on TCBS. Then, the isolates were identified by biochemical reactions.

Results: Vibrio parahaemolyticus and other species of vibrio were present in (6) 1.5% and (10) 2.4% of samples respectively.

Conclusion: The Results of the present investigation indicate the fact that there is a need to improve hygienic condition of seafoods in Isfahan.

Keywords: Vibrio, Vibrio parahemolitycus, contamination, seafoods, fish, shrimp
Use of interdelta polymorphisms of *Saccharomyces cerevisiae* strains to monitor the population evolution during wine fermentations

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**Aims:** Use of interdelta polymorphisms to monitor the population dynamic of *Saccharomyces cerevisiae* strains during fermentation of grape juice.

**Methods and Results:** Three industrial *S. cerevisiae* wine strains were screened for interdelta polymorphisms using a set of three pair of primers (delta1-delta2; delta12-delta2 and delta12-delta21). PCR-amplification reactions originated different fragment patterns for each pair of primers and the pair delta12-delta2 exhibited the best resolution and discriminatory power. This pair of primers (delta12-delta21) was thus selected and used to follow the yeast population evolution during a mixed fermentation performed in synthetic grape juice and inoculated with similar amounts of each strain. All strains exhibited exponentially growth during the first three days of fermentation, attained maximal cell densities ranging from 10^6 to 10^7 CFU ml^-1 and kept these high density cell values throughout the stationary growth phase (from 3rd to 10th day) without significantly changing their relative population proportion.

**Conclusions:** Population quantification demonstrated that these three *S. cerevisiae* strains were able to grow together during the whole fermentation process, thus indicating that each strain, albeit at different levels, should influence the chemical composition and final flavor of wine.

**Significance and Impact of the Study:** Molecular typing of *S. cerevisiae* strains by PCR-amplification of DNA delta sequences is a reproducible, strain-specific and simple method that can be successfully used to monitor the population dynamic of wine fermentations.

**Keywords:** strain-specific primers; interdelta polymorphisms; mixed starter cultures; wine fermentations; yeast population dynamics; molecular methods.

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Use of the E-beam radiation to diminish the late blowing of cheese slices

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The late blowing of cheeses is a spoilage caused by the growth of *Clostridium tyrobutyricum* and related species. It is due to the butyric fermentation of lactate by these organisms accompanied by the gas generation. The products more frequently involved are both the long ripening time cheeses make with enzymatic curd and processed cheeses. This phenomenon is relatively uncommon if the product is stored under refrigeration because of the mesophilic condition of clostridia but their frequency increases markedly when a temperature abuse occurs if the environmental conditions are favourable for the growth of clostridia even if a lactic acid microflora is present. This paper is an attempt to prevent the growth of clostridia responsible for the late blowing to statistically negligible levels by applying E-beam irradiation.

Cheese slices of about 5 month of ripening acquired in the market were contaminated with spores of a *C. tyrobutyricum* suspension at the rate of 10^8 spores/g and then vacuum packed. Samples were submitted to different doses of radiation in an E-beam irradiation plant. The absorbed radiation was controlled by the cellulose dosimeters. After treatment, the bacterial survivor count was made by pour plate method and the sensory quality was estimated using preference and descriptive tests. The colour and texture were also estimated by instrumental techniques.

The rate of clostridia death by radiation was fitted to a first-order reaction according to the equation log c.f.u/g = - 0.5645 dose + 7.3171, from which a value D = 1.77 kGy was determined. A treatment of 3 kGy will reduce a 99%, decreasing a hundred-fold the spoilage possibility.

As the non-sporeforming microflora present in the cheese (mainly lactic acid bacteria) is much more radiosensitive than the spores, a 3 kGy treatment also produced a very important kill of these organisms, in such a way that the mesophilic aerobic counts was reduced from 7.69 log c.f.u./g in non-treated samples to 5.60 log c.f.u./g, 3.76 log c.f.u./g and 2.53 log c.f.u./g after the application of 1, 2 and 3 kGy, respectively. The counts of non-sporeforming bacteria were increasing afterward to reach normal values (> 7.00 log c.f.u./g) after 1 – 3 months of storage at 4 °C.

To study the effect of E-beam radiation on the sensory properties of the processed cheese, many slices were treated at doses of 1, 2 and 3 kGy and analysed by both sensory and instrumental methods. Some significant differences (p < 0.05) were found between non-treated samples and those treated with 3 kGy dose. Differences were found in the flavour just after treatment and after 14 days of storage but these differences were minimised during storage and after 28 days were not detected. Differences were found at 28 days of storage even when 1 kGy was applied. However, samples were considered by testers as acceptable for consumption. The instrumental analysis of the colour showed that irradiation gave rise to light changes in the colour. In general, samples showed a trend to approach to the red (increase *a*^*b* values) and move away from the yellow (decrease *b*^*L* values). The luminosity (*L*) was not affected.
Wine ecological practices increase the chromosomal polymorphism of yeasts

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Introduction and Experimental Procedures. Yeasts taking part in the aging sherry-like wines elaboration in Montilla-Moriles area (Córdoba, Southern Spain) belong to different varieties of \textit{Saccharomyces cerevisiae}. This microbe has been characterized, emphasizing the populations stability, the sexual isolation and the low level of genetic polymorphism (1, 2). Besides, in that region, since 1999, an "ecologic" aging sherry-like wine is produced by using natural procedures friendly with the environment. In this work we study the chromosomic polymorphism level of aging yeast populations present in this type of wine, made under special conditions, remarkably different to those used by traditional methods. Wine samples were taken from aging barrels belonging to a wine prepared according ecological procedures. Yeasts were isolated and selected from different aging stages (200 strains in total). The identification of the isolations was made on the basis of physiologic, metabolic and molecular trials. We performed karyotype electrophoretic determination (PFGE), which separates DNA fragments (250 to 2700 Kb), equivalent to the yeasts chromosome size (3), by using Bio-Rad material (CHEF-DRIII Systrem and GelDoc-3000 chamber).

Results and discussion. Microscopic observation of cellular morphology of yeasts, together with some biochemical tests allowed to rule out 40 strains that did not belong to the \textit{Saccharomyces} genus. The remaining 160 \textit{Saccharomyces} strains were classified on the basis of different informative assays. The results (all strains formed film, high sensitivity to lithium -no growth in 50 mM LiCl-, sucrose fermentation) showed the predominance (95 %) of \textit{Saccharomyces capensis} variety. This percentage is similar to the corresponding one of traditional wines of the same region.

We performed total DNA electrophoresis (PFGE) to 60 aging strains and we found 4 different karyotypes (I, II, III, IV). The comparison between these karyotypes with the marker of \textit{S. cerevisiae} YNN295 displayed the following relevant differences: disappearance of chromosome XII, appearance of a new chromosome over the XV, appearance of one chromosome between IV and XV, chromosomes III and IV were merged in karyotype II was the predominant at all the stages and it was the only pattern found at the end of the aging period.

Conclusions. The chromosomic polymorphism level detected in ecological wine was higher (7,5 %) than the corresponding to the traditional wines (5%), which is in accordance to the less restrictive conditions and larger biodiversity during ecological vinification. The adaptation and predominance of one population line (karyotype II) of yeasts during the aging period suggests the putative feasibility of that line as selected starter to be used at industrial level. The potential risk due to the inusual presence of undesirable strains of non \textit{Saccharomyces} yeasts during the aging wine makes reasonable to develop strategies at different levels (physiscal, chemical, microbiological, building design) in order to elaborate a healthy and quality product maintaining sustainable winemaking practices.

References

Keywords: Wine, ecological practices, chromosomic polymorphism, yeast.
Yeast, beer and fermentation: an opportunity to involve young students in biotechnology.

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This biotechnology and its applications have an important role in economics, industry, health, nutrition, on environment and more science fields. However, our society has a negative perception of the biotechnology. In order to change this situation it is essential to improve biotechnological dissemination with secondary school students, our future society.

Designing a simple research work to secondary school students related to biotechnology could be complicated because it is difficult to correlate it with a specific subject, chemistry, biology, technology, etc. Furthermore, secondary school laboratories are not usually provided with adequate facilities and equipment. Finally, introducing secondary school students to the safety and conduct rules working in a microbiology laboratory is necessary.

We have prepared and developed some experiments focused on growing microorganisms and fermentation revision. In this way, cellular morphology and metabolism were studied; yeast replication and glucose catabolism were also measured and compared in two different Saccharomyces cerevisiae strains: CECT 1318, CECT 1383. Both are non pathogenic yeast with important significance in the beer industry. In addition, they are the most widely utilized yeast strains in undergraduate laboratories. Experiments were carried out in university laboratories with several groups of secondary school students (16 to 18 years old), each group spent two days in performing these experiments.

This microbiologic-educational approach has involved science secondary school students. The approach was based in microbial physiology and fermentation technology with a trouble-free communication and debates about biotechnology with laboratory researchers. Every simple experiment was focused in showing how indispensable the biotechnology and its application are in our society. Moreover, laboratory exercises are directed to secondary school students to modify their general negative opinion about biotechnology.

This project has increased the relationship between science secondary school (students and teachers) and university teachers and researchers. Working together, secondary school and university will improve microbiology and biotechnology knowledge in the whole of the society.

Keywords: Biotechnology, fermentation, education and secondary school students.

A Biodegradation Study of Forest Biomass by Aspergillus niger Strain Showing Correlation Between Enzymatic Activity, Hydrolysis Percentage and biodegradation Index

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In the current time, the importance of alternative energy source has become even more necessary not only due to the continuous depletion of fossil fuel stock but also for the safety and better environment. With an inevitable depletion of the world’s energy supply, there has been an increasing worldwide interest in alternative sources of energy. The production of liquid biofuels from lignocellulosic biomass can significantly reduce the world dependence on oil, so it has become a research area of great interest to many governments, academic groups and companies. Today, it is possible due to advances in biotechnology to propose the inexpensive production of ethanol as biofuel. However, the cost of ethanol as an energy source is relatively high compared to fossil fuels. A potential source of low cost ethanol production is to utilize lignocellulosic materials such as forest biomass, crop residues and grasses, etc. due to their abundance, low cost, easy availability and regenerative capacity for bioconversion to sugars. In the present investigation, an attempt has been made to utilize forest waste as substrate for its degradation by potential cellulolytic and hemicellulolytic microorganisms isolated from soil under solid state fermentation and to enhance their rate of hydrolysis which is a key step for its bioconversion to ethanol. Different substrates used for bioconversion were wood chipping of Pinus roxburghii, Cedrus deodara, Toona ciliate and Celtris australis. SSF of biomass moistens with water by A. niger has produced high level of hydrolytic enzymes cellulase and xylanase which inturn led to efficient hydrolysis and thus enhanced biodegradation index of different lignocellulolytic substrates. Highest extracellular enzyme activity of 220 U/g by A. niger was shown in pretreated Celtris australis wood resulting in 6.5% hydrolysis and 6.99 BI. The lowest BI of 1.40 was observed in untreated saw dust of Cedrus deodara having the least release of cellulase + xylanase i.e.238 U/g of dry matter and 1% hydrolysis. It has been found in the study that SSF of forest biomass by A. niger increased remarkably when water is replaced by modified BSM as moistening agent. In modified basal salt medium (BSM) mediated degradation of forest waste with A. niger extracellular enzyme activity was increased to 4089 U/g of dry matter resulting in higher BI of 32.42 and 20% hydrolysis of C. australis wood. Statistically a positive correlation has been shown between these three factors i.e. enzyme activity, BI and percent hydrolysis of forest biomass by A. niger proving that these are directly proportional to each other.
Ability of xylitol production by new yeast strains

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Activated carbon production from brewer’s spent grain lignin

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Activated carbons are adsorbents that are industrially used in multiple processes for product separation and purification, and for the treatment of liquid and gaseous effluents. Despite its frequent use in the water and waste industries, activated carbons remain an expensive material. In view of the high cost and the tedious procedures for the preparation and regeneration of activated carbons, there is a continuing search for low-cost potential adsorbents. The preparation of activated carbons from lignin is an attractive way of giving added value to this material, which is mainly used as in-house fuel for the recovery of both energy and residual inorganic matter. Over the past few decades, some works have been done on the activation of agricultural lignocellulosic waste materials to carbons, due to their low cost and high availability. Nevertheless, there is not any literature report about the activated carbon production from brewer’s spent grain (BSG) lignin. Use of BSG lignin as starting material for activated carbon production is interesting because BSG (the main brewery by-product) is produced in large amounts during all year, and is a lignin-rich material. Lignin can be converted in activated carbon by physical or chemical activation, the last one being more amply used than physical activation, because it requires lower activation temperatures and gives higher product yields. The purpose of the present work was to prepare activated carbon from BSG lignin, by chemical activation using phosphoric acid as impregnating agent, and to examine the influence of preparation conditions (acid/lignin ratio and carbonization temperature) on the textural characteristics of the materials produced (surface area, volume of pores, and pores size distribution) as well as on its adsorption capacities.

Chemical activation of BSG lignin using phosphoric acid as impregnating agent was performed at various acid/lignin ratios (1, 2, or 3 g/g) and carbonization temperatures (300, 450, or 600 °C), according to a 23 full factorial design. The resulting materials were characterized (regarding the surface area, volume of pores, and pores size distribution), and used for detoxification of the BSG hemiicellulosic hydrolysate, which consists in a mixture of sugars, phenolic compounds, metallic ions, among other compounds. BSG carbons presented BET surface areas between 33 and 692 m²/g, and volume of pores between 0.058 and 0.453 cm³/g, which generally consisted in micro and mesopores. Adsorption capacity also varied to each carbon, according to the used activation condition. However, all of them showed high capacity for adsorption of metallic ions, mainly nickel, iron, chromium and silicon. In most of the cases, the BSG carbons efficiency for removal of these metals was higher than that obtained when using a commercial carbon sample. Phenolic compounds concentration and color were also reduced by using these sorbents, and the sugars content was practically not affected, which is beneﬁc if the hydrolysate use in bioconversion processes is desired.

The present work allowed to conclude that it is possible to produce activated carbons with good efﬁciency for phenolic compounds and metallic ions removal (mainly Ni, Fe, Cr, and Si), by chemical activation of the BSG lignin. The adsorption capacity of the carbons compared well and even favorably with that of a commercial activated carbon, suggesting that they have potential to be successfully used in detoxification processes in substitution of commercial sorbents. Regarding to the preparation of these activated carbons, an impregnation ratio and activation temperature of 3 g H3PO4/g lignin and 600 °C, respectively, was the best combination of operating conditions leading to activated carbons with good capacity for adsorption of different toxic compounds. Acknowledgements: CAPES, FAPESP and CNPq (Brazil).

Keywords: brewer’s spent grain; lignin; chemical activation; activated carbons; hemiicellulosic hydrolysate.
Acute toxicity evaluation of several compounds involved in fossil fuels biodesulphurisation studies

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The increasing use of fossil fuels has led to increased emissions of sulphur oxides into the air, which is a major cause of acid rain. Legislation already adopted in 2009 stipulates that the maximum level of sulphur allowed in fuels is only 10 ppm. The process of hydrodesulphurization (HDS) used in refineries is based on very expensive physico-chemical techniques, and has limitations in the removal of organic sulphur. As for stricter legislation on the maximum levels of sulphur in fossil fuels, the most HDS recalcitrant compounds needs to be removed. This implies an increase in the intensity of the physical-chemical treatment and inherently its associated costs. As a result, the recalcitrant compounds to HDS represent a significant barrier to the achievement of very low levels of sulphur in some petroleum fractions.

The alternative to the physical-chemical treatment could be the use of biological processes (biodesulphurisation) which is more effective for the desulphurization of fossil fuels, especially as the removal of sulphur covalently bound to organic matrices. The biodesulphurisation (BDS) occurs in more mild conditions of operation under conditions of atmospheric pressure and temperature, giving greater specificity of reaction due to the nature of the biocatalysts, not requiring molecular hydrogen. Thus, in the last 15 years there has been an increase of studies involving the use of microorganisms with the ability to specifically remove the HDS recalcitrant sulphur compounds.

Several model compounds such as dibenzothiophene (DBT), DBT sulphone or benzothiophene (BT) are used in BDS studies to characterise organic sulphur in coal, coal tars and crude oils. The desulphurising microorganisms are able to remove the sulphur atom from these compounds and use it in their metabolism. However, such compounds are very toxic to the cells. The aim of this work was to evaluate the toxicity of several compounds used in BDS studies, such as DBT and its derivatives and organic solvents used to dissolve these hydrocarbons, to two typical desulphurising strains, namely: *Gordonia alkanivorans* strain 1B and *Rhodococcus eritropolis* strain D1.

The toxicity bioassays evaluated the inhibitory effect of the studied compounds to the described bacteria by measuring the respiration rate (mg O₂/l) under defined conditions in the presence of different concentrations of those compounds. The inhibitory or toxic effect of each chemical at a specific concentration is expressed as a percent of the baseline respiration rate. From these results the several IC₅₀s were estimated and are described in Table 1. These toxicity values showed that strain 1B was less sensitive for almost all of the hydrocarbons, which is an important advantage considering the desulphurisation of fossil fuels process. On the other hand, strain 1B was more sensitive to dimethylformamide (DMF), a typical solvent used in BDS studies. However, a good correlation can be observed between IC₅₀-1B versus IC₅₀-D1 (IC₅₀-D1 = 0.504 x IC₅₀-1B + 2.84; r² = 0.908, p < 0.05).

### Table 1. Acute toxicity of several compounds involved in the biodesulphurisation studies.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>1B Toxicity Bioassay</th>
<th>D1 Toxicity Bioassay</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>BT</td>
<td>1.61 mM 0.904</td>
<td>1.15 mM 0.963</td>
<td>1.4</td>
</tr>
<tr>
<td>Ethanol</td>
<td>10.56% 0.995</td>
<td>10.52% 0.992</td>
<td>1.0</td>
</tr>
<tr>
<td>DMF</td>
<td>6.56% 0.963</td>
<td>9.91% 0.940</td>
<td>0.7</td>
</tr>
<tr>
<td>2-HBP</td>
<td>0.62 mM 0.962</td>
<td>0.52 mM 0.950</td>
<td>1.2</td>
</tr>
<tr>
<td>DBT</td>
<td>44.35 mM 0.906</td>
<td>24.21 mM 0.913</td>
<td>1.8</td>
</tr>
</tbody>
</table>

IC₅₀ = inhibitory concentration that causes an inhibition of 50% of the bacterial baseline respiration rate

*IC₅₀-D1 / IC₅₀-1B is the Sensitivity Factor (R).

This study also shows the high toxicity of 2-Hydroxybiphenil (2-HBP), the final microbial product from DBT desulphurisation, to both desulphurising microorganisms tested. The physiological response of 2-HBP concentration was also studied in a bioreactor system using both strains.

*Keywords*: biodesulphurisation; fossil fuels; *Gordonia alkanivorans*; *Rhodococcus eritropolis*; acute toxicity

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Alternative method for biological airborne agents detection in only few hours / Innovative microbial air sampler.

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In the context of environmental contamination control and bio-sample preparation, Bertin Technologies (France) has developed a range of laboratory equipments based on new technologies dedicated to collection and sample preparation. One of these technologies is dedicated to the monitoring of airborne bio-particles. The goal is to propose a sampling method compatible with Rapid Microbiological Methods in order to get rapid, reliable and specific data on airborne biological agents and go beyond impaction method limits.

This technology aims at going beyond the traditional impaction method (impaction on agar plates) in terms of time-to-result, more information than only cultivable flora (VNC, viruses, allergens…) and no saturation of the collection media.

With the Coriolis® technology, many studies have been carried out for the sampling of airborne bio-particles to detect bacteria, virus, pollens, allergens or non-cultivable pathogens with rapid microbiological methods as PCR analysis (*Pneumocystis*, Respiratory Syncytial Virus (RSV), bacteriophage, *Legionella*, *Stachybotrys chartarum*…). The content of the speak could include:

- the technology qualification validated by ISO14698-1 (Health Protection Agency HPA, Porton Down, UK)
- the application fields (pharmaceutical industry, food industry, hospitals, aerobiology…)
- the cases studies in production site, hospital environment, clean rooms…

*Keywords*: air sampler, environmental contamination control, monitoring of airborne bio-particles.
Amino acid uptake profiling of *Streptomyces lividans* batch fermentations

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Streptomyces are worldwide used for the commercial production of antibiotics (e.g., by CIPAN s.a.) and industrial enzymes (e.g., Danisco-Genencor International). Recently several species of Gram-positive bacteria are being tested as host for the production of heterologous proteins due to their ability to efficiently secrete proteins in the culture medium. Among them *Streptomyces lividans* is considered an interesting host for the secretory production of heterologous proteins [1]. To obtain a good secretion yield of heterologous proteins, the availability of suitable nitrogen sources in the medium is required. Often, casamino acids are added to the medium for this purpose. The amino acids in this nitrogen source not only act as building blocks for the biomass but also play an important role in the biosynthesis of the heterologous protein. The availability of different amino acids and varied cellular preferences for them can have a dramatic effect on the protein production. Ultimately, the goal is to perform metabolic flux analyses to unravel these interactions. In this work, we start with amino acid profiling for both the wild-type and recombinant strain in various batch conditions and the qualitative discussion of these results to get a first insight in the amino acid uptake mechanisms and regarding the influence of heterologous protein overproduction/secretion on these uptake mechanisms.

For this work, batch experiments with *S. lividans* 66 strain TK24 (John Innes Centre, Norwich, UK) and *S. lividans* pIJ486 rmTNF-α are performed in a computer controlled 5L benchtop bioreactor (BioFlo 3000, New Brunswick Scientific, USA). Minimal liquid NMMP, containing casamino acids and ammonium as nitrogen sources, is used as medium [2]. Samples collected periodically are evaluated for biomass and extracellular metabolites. Amino acids are measured using the Phenomenex EZ:faast™ amino acid analysis kit developed for GC-MS. Using a Perkin Elmer Autosystem XL-Turbomass Gold GC-MS and norvaline as internal standard, this method is able to analyze the samples for all amino acids present in the casamino acid mixture, except for arginine and cysteine.

An example for the wild-type strain is illustrated in Figure 1. After a lag phase (not shown), exponential growth is initiated during which glucose, ammonium and amino acids are co-metabolized. Clearly, differences in preference and uptake rate for (groups of) amino acids are observed. Glutamate and aspartate (correlated to the pathways of ammonium assimilation) are rapidly consumed, whereas the remaining amino acids, e.g. the branched chain amino acids, are taken up more slowly. Remarkably, the alanine (Ala) concentration first increases, most likely due to D-Ala liberation during cell wall synthesis. Afterwards, however, the total amount of (D-) Ala is rapidly consumed.

![Figure 1. Glucose (○), ammonium (●) and amino acid uptake during the exponential growth phase (10-30 hrs) in a batch fermentation (60 hrs) with NMMP medium initially containing 55.5 mM glucose, 30 mM ammonium and 5g/L casamino acids.](image)

The profiles of the wild-type and recombinant strains shall be put against each other. Moreover, experiments with high levels of casamino acids are being conducted and will be presented.

Acknowledgements

This research is supported in part by the Research Council of the Katholieke Universiteit Leuven (projects OT/09/25 and EF/05/006 OPTEC Optimization in Engineering), the Belgian Program on Interuniversity Poles of Attraction, initiated by the Belgian Federal Science Policy Office and the Fund for Scientific Research Flanders (FWO-Vlaanderen) (project G.0352.09 and Postdoctoral Fellow K. Bernaerts).

Keywords amino acid uptake; *Streptomyces lividans*; heterologous protein production

References

ANN-based Software Sensor for Emulsification Activity Estimation in Biosurfactant Production Process by Candida lipolytica UCP 0998

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Artificial neural network (ANN)-based software sensors with one hidden layer were developed to estimate emulsification activity of emulsions water in hexadecane, water-in-corn oil and water-in-canola oil in biosurfactant production process by Candida lipolytica UCP 0988. The input variables used were pH and dissolved oxygen. The data sets required to train, validate and test the software sensors were obtained from experiments carried out in a 5L bioreactor, under different temperature and agitation conditions. Corn oil was used as carbon source and natural sea water diluted a 50%, supplemented with urea, ammonium sulphate and potassium dihydrogen phosphate, was used as low cost basal medium. The Levenberg-Marquardt algorithm, in conjunction with Bayesian regularization was used in trainings. The root mean square error (rmse) and the global determination coefficient (Rg2), among others indexes, were used to compare model performances. On-line emulsification activity estimation results are within an acceptable variation of 3% of the experimental values. Global coefficient of determination higher than 0.92 indicated excellent agreement of the neural network model with experimental validation and test values, obtained for emulsification activity.

Keywords: artificial neural network, software sensor, emulsification activity, biosurfactant, bioemulsifier, Candida lipolytica.

Antibacterial activity and probiotic properties of Algerian strains of lactic acid bacteria

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During the last 2–3 decades, attempts for improving the human health status, are focusing on ways for modulating the indigenous intestinal flora by live microbial adjuncts, now called “probiotics”. The most typical active components of probiotic products are lactic acid bacteria, including bifidobacteria, lactobacilli and enterococci.

In this study, the probiotic potential of five selected bacteriocinogenic strains of lactic acid bacteria (L. lactis subsp. lactis, Ec. faecalis and Lb. paracasei subsp. paracasei) was investigated. The strains were previously revealed active towards listerial strains and able to produce bacteriocins-like substances. The spectrum of their antibacterial activity was determined, including strains of Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium, and Bacillus cereus using the spot and the well diffusion methods. They were examined for resistance to pH 1, 2, 3 (in presence of pepsin), pH 8 (in presence of pancreatin), and to bile salts (0.5, 1 and 2%). From the results obtained in vitro, only the Lactobacillus and the Enterococcus strains were able to survive at pH 1 or in the presence of pepsin, while all were unaffected by pH 3, pancreatin and bile salts. Strains exhibited variable antibacterial activity; they inhibit all the Gram positive strains. However only Lb. paracasei subsp. paracasei inhibit the Gram negative bacteria tested (Escherichia coli and Salmonella typhimurium). The inhibition diameters obtained are between 8 mm and 12 mm. These five strains were therefore found, in vitro, to possess desirable probiotic properties.

Keywords: Lactic acid bacteria, Probiotic; Antibacterial activity; Survival.
Antifungal potential of *Cladosporium cladosporioides* (Fres) De Vries metabolites in reduction of coffee rust (*Hemileia vastatrix* Berk & Br.)

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Brazil is the world biggest coffee producer, exporter and the second largest consumer. Rust is the most important disease of coffee and chemical processing is the main control strategy, but has disadvantages such as toxicity to farmers, high application cost and risks to environmental, and fungi resistance development. Therefore, there are motivation to search for alternative methods of controlling the disease, based mainly on the use of microorganisms and their metabolites. Research developed shown disease association to coffee fruits and a *Cladosporium* genus is always related with good coffee quality. Complementary studies and wide literature review about the fungus showed that it presents characteristics of GRAS (Generally Regarded as Safe). This fungus was collected and identified as *Cladosporium cladosporioides* (Fres) De Vries. This present research showed that the extract capacity obtained from a selected isolate of *Cladosporium cladosporioides* (Fres) De Vries, after testing extractors from four different extracts (methanol, ethanol, DMSO and ethyl acetate) on *Hemileia vastatrix* Berk & Br. spores germination, obtained from contaminated leaves. It was observed a germination inhibition by ethanol extract in 60%. So it may conclude that metabolites produced by fungus are efficient in important fungi phase development reduction in coffee rust disease. A natural fungicide development based on fungus metabolites is subject of the researches that are in development at this moment and can be alternatives to chemical disease controls.

Assessment of the use of biological material on technological development – a patent approach

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Microorganisms have been used in a broad range of biotechnological processes for a very long time and their use has intensified in the last decades as a consequence of advances in handling and characterization techniques for microorganisms and their products. The intellectual property rights of a large number of these technologies are protected by patents. The use of the patent system requires the disclosure of the technology to be protected with the purpose of stimulating the development of new technologies, or the improvement of existing ones. Information related to patent applications is made available, among other means, through free online databases from several national or regional patent offices. In the case of patent applications that involve the use of biological material, the deposit of a sample of this material in a culture collection can be required by the patent office so that the technology is also made available as a living organism. The use of biological materials such as microorganisms and cell cultures for developing technologies in health, food, energy, environmental, industrial and agricultural areas was assessed through the Epodoc database. International and European Patent Classification codes related to technological fields such as pharmaceutical preparations, fermentation and waste treatment were identified in this study. Our results showed that the total number of worldwide biotechnology-related patents indexed in the database to describe the manufacture of food and pharmaceutical preparations was about 24,000 and 265,000 documents, respectively. The total number of documents related to waste treatment was about 370,000 and the number of documents describing bioprocesses for the manufacture of organic compounds was about 357,000. We also found that approximately 630,000 documents describe microorganisms and processes related to their handling. These results illustrate the importance of microorganisms for technological development in many fields and suggest that the patent system is an important source of information for further advances in the studied areas.

**Keywords**: microorganisms, patents, technological information
Bacteria exhibiting antimicrobial activities; screening for antibiotics and the associated genetic studies
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In the search for new antibiotics, the genus *Bacillus* is an excellent place to look. *Bacillus* species produce a large number of antibiotics representing at least 25 different basic chemical structures. Different *Bacillus* species have been isolated and identified i.e. *Bacillus subtilis*, *B. amyloliquefaciens*, *B. cereus*, *B. firmus*, *B. licheniformis*, *B. mycoides* and *Paenibacillus polymyxa*. Identified strains showed interesting biological activities e.g. inhibiting the growth of clinical isolates (*Klebsilla* species), strong antifungal and antialgal activities and high toxicity against *Artemia* sp., while their TLC and HPLC profile showed an impressive chemical diversity. All the strains were able to produce number of peptides (surfactins, iturins, fengycins, subtilin and subtilosin) in different combinations. Over 50 compounds were isolated and identified. Standard identification data and records in term of measured values for NMR and MS are presented in this work. The approach of total secondary metabolites isolation led to the isolation of new natural bioactive secondary metabolites with various biological activities: heptyl-1-hydroxyquinolin (NEW), indol-2-oxoacetamide (NEW), oxopentyl-acetamide (NEW) and a number of cyclic de-peptides cyclo(His, pro), cyclo(His, Leu) (NEW) and cyclo(Tyr-Pro) (NEW). Other worth of noting findings and observations included the isolation of bacillamide and macrolactins (previously known to be exclusive products from marine bacteria) from terrestrial *Bacillus* species, isolation of setonycin, macroclacin and quinoline derivative (known as typical products from streptomycines) from *Bacillus* species and finally, the isolation of heptyl-1-hydroxyquinolin (NEW) as a new class of antibiotics. Presence of *sboX* gene was not correlated with subtilosin production, however, subtilosin and *sboX* were confirmed in *Bacillus amyloliquefaciens* for the first time. As a conclusion, this study has not only provided new bioactive compounds but also a comprehensive standard profile of *Bacillus* secondary metabolites for convenient dereplication. It was also possible to add new metabolites to these records. These bioactive new products are luxuriant materials for further research work to validate proposed medical or biotechnological applications and their physiological and ecological roles.

**Keywords:** Bacillus species, novel antimicrobial agents, heptyl-1-hydroxyquinolin, *sboX* gene.

Biocidal potential of essential oils of *Piper aduncum, Piper hispidinervum* and *Syzygium aromaticum* on important pathogenic and toxigenic microorganisms important for food.
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The biocidal potential of *Piper hispidinervum, Piper aduncum* and *Syzygium aromaticum* essential oils obtained from the hydrodistillation were tested against *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Aeromonas hydrophila*, *Candida albicans*, *Cladosporium cladosporioides*, *Penicillium citrinum*, *Penicillium brevicompactum*, *Penicillium solitum* and *Aspergillus flavus*. The best results were obtained with *Syzygium aromaticum* oil, that was able to inhibit all microorganisms tested at a concentration ≤ 125 mg / mL. *Piper aduncum* and *Piper hispidinervum* oils not show satisfactory results in comparison to *Syzygium aromaticum* oil.

**Acknowledgements** The support FAPEMIG, CNPq e Fundação André Tosello is gratefully acknowledged

**Keywords** essential oils, foodborne, antimicrobial
Biocompatibility Assessment of PHB, Random and Block copolymer of P(3HB-co-3HV) produced by Paracoccus denitrificans

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The aims of this study were to evaluate the potential use of biodegradable thermoplastics focusing on polyhydroxyalkanoates, PHA; produced from microorganisms. Previously, we have successfully developed a metabolic reaction based system for the production of poly (3-hydroxybutyrate-co-3-hydroxyvalerate), P(3HB-co-3HV), in fed-batch culture by Paracoccus denitrificans ATCC 17741. Two types of P(3HB-co-3HV), random-P(3HB-co-3HV) and block-P(3HB-co-3HV), can be produced at arbitrary values of HV content varying from 0 to 90 mol%. In the present contribution, films made of poly(3-hydroxybutyrate) (PHB), random-P(3HB-co-3HV) consisting of 5%, 12%, 26%, 53%, 60%, 72% and 80% of HV and block-P(3HB-co-3HV) consisting of 12%, 19%, 41% and 50% of HV, respectively, were prepared and evaluated their biocompatibility in vitro against three types of recommended mammalian cells: L292 mouse fibroblast cells, human dermal fibroblast cells and Saos-2 human osteoblast cells. The mechanical integrity of these PHB and P(3HB-co-3HV) films and the characteristic of film surface were changed in accordance with the content of 3HV in random-P(3HB-co-3HV) and block-P(3HB-co-3HV). The random-P(3HB-co-3HV) possessed smooth surface whereas PHB and block-P(3HB-co-3HV) possessed the surface rich in crystallites as observed by SEM and SPM analysis. Moreover, the random-P(3HB-co-3HV) possessed mechanical properties significantly different from those of block-P(3HB-co-3HV). All biodegradable films showed excellent biocompatibility for the attachment and proliferation of three types of mammalian cells. The indirect cytotoxicity assay and the production of transforming growth factor (TGF)-beta-1 and interleukin-8 (IL-8) were investigated. In particular, the 2-D LC-MS system was introduced in this study for preliminary investigation based on proteome analysis of abundance cellular proteins in depth aiming at determination of cell-biomaterial-interaction. Altogether, this in vitro study demonstrates biodegradable and biocompatible properties of our produced PHB and P(3HB-co-3HV) and their feasibility for biomedical applications.

Keywords Biodegradable polymer, Random copolymer, Block copolymer, Biocompatibility, P(3HB-co-3HV)

Bioconversion of wheat straw to value added cattle feed by RCK –1 fungal isolate

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Microbial conversion of agricultural wastes is an environmentally benign and practical method, which has the potential of increasing nutritional value for production of energy rich cattle feed. Many white rot fungi have been investigated for their capability to degrade lignin, the major recalcitrant polymer in plant cell wall, and eventually to increase the digestibility of the fermented agroresidues in ruminants. Majority of them degrade lignin simultaneously with carbohydrate content or degrade larger amount of carbohydrate with smaller or negligible degradation of lignin leaving behind the fermented product deficient in energy and nutrition, while some of them have been found capable of degrading lignin selectively.

In the present work a new fungal isolate RCK–1, which has been identified based on Internal transcribing sequence (ITS) of Crinipellis sp., was chosen for converting wheat straw for cattle feed production based on its faster rate of colonization of lignocellulosic substrates and higher rate of production of ligninolytic enzyme. The moistened wheat straw (10 g) was inoculated with the fungus and incubated for 15 days at 30°C and 60% relative humidity. Samples were drawn periodically and then analyzed for compositional changes, laccase activity, crude protein content, biomass concentration and in-vitro dry matter digestibility (IVDMD). Bioconversion of wheat straw was optimized and scaled up in stages of 2kg, 5kg, 25 and 30 kg in shallow beds. Similar scale-up in deep bed reactors was carried out in two stages: 150 g in a 7 liter and 60 kg level in 1200-liter bioreactor. The exit CO2 and O2 concentrations from the reactor was monitored in real time.

The maximum SSF efficiency (15.38%) was observed on 9th day with 18.36% degradation of lignin and reduction of cellulose and hemicellulose by 10.3% and 28%, respectively. The fungal fermentation of wheat straw resulted in a feed with improved IVDMD up to 10%, protein up to 37.5% and crude fat up to 65%. Maximum evolution of CO2 on 3rd day of incubation correlated with maximum decrease in O2 level and fungal biomass was interestingly found to increase thereafter till 7th day. Lactose titre was maximum on 5th day of incubation.

The fermented feed was evaluated for its toxicity, palatability and digestibility in a rat model ( Holtzman strain). Mycotoxin studies on treated feed showed trace amounts of aflatoxin B1, B2, G1 and G2 (< 0.5 μg/kg). The dry matter (DM), of substrate treated with Crinipellis sp. possessed higher digestibility compared to the raw substrate (P<0.01) DM, Organic matter (OM), Crude fiber (CF) and total carbohydrates were found to be 56.18%, 58.99%, 52.01% and 58.01% respectively, for treated feed. The digestibility of ADF (43.13%) and cellulose (45.02%) was better (P<0.01) on T3 diet. The digestibility of NDF (P<0.01) and hemicellulose (P<0.05) on T3 diet was 57.9% and 63.29%, respectively, which is significantly higher than control diet. The activity of Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) was comparable on T1 and test diets T3. The Lactate dehydrogenase (LDH) activity was significantly (P<0.01) lower and comparable between rats fed T1 diet and T3 diet. No gross pathological lesions were observed in the vital organs (brain, heart, lung, liver, kidney, spleen, pancreas, stomach, intestine and testis) of rats feed with all diets.

Keywords Solid-State Fermentation, White Rot Fungi, Lignin Degradation, Laccase, IVDMD, Bioreactor, Aflatoxin
Biofouling community of a lubrication oil tank from a supply vessel

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Biofouling of industrial applications is of significant concern regarding economy, human health, environmental considerations and safety. The diversity, succession and community stability of the microorganisms in industrial applications are mainly unknown. There are to our knowledge no published results of the diversity, community stability and succession of such communities in lubrication and hydraulic systems.

The microbial diversity in a sample of flakes of paint from the oil tank of a supply vessel was investigated. The paint samples were covered by a dehydrated biofilm revealed by scanning electron microscopy (SEM) and contained fungi, bacteria, fungal spores and yeasts. However it was not possible to extract DNA directly from the paint sample and culturing with lubrication oil as the sole source of carbon was necessary. The dominating microorganisms of the culture were revealed by cloning and sequencing of the 16S rRNA gene for bacteria and 18S rRNA gene for fungi, and identified by searching the NCBI Blast database. The culturing was performed in a CDC biofilm reactor and we could therefore analyze biofilm, oil phase and water phase. Both bacteria and fungi were found and identified.

Of the bacteria, we identified *Variovorax* sp, *Shewanella* sp, *Achromobacter* sp, *Pseudomonas* sp, *Delftia* sp and *Acinetobacter* spp, of which the two latter species were the most abundant. Of the fungi, we identified *Phialocephala* sp and *Fusarium* sp.

The fungi were the visually most abundant organisms on the SEM micrograph of the paint flakes. After 8 weeks biofilm coupons were analyzed by SEM, and fungi were not observed. However, the fungi were present in the liquid-air interface in the CDC biofilm reactor at all times. The results indicate that fungal growth might be repressed by bacteria in a circulating system.

Fungi in oil and fuel storage tanks could be a serious threat to health of workers exposed to bioaerosols, and the fungi could cause extensive degradation of petroleum hydrocarbons, thus reducing the oil's fuels quality, and biomass might clog filters and nozzles leading to malfunctioning of machinery. Bacteria may be the organisms of greatest concern regarding biofouling in industrial applications due to their ubiquitous nature in all sorts of man made and natural settings.

Denaturing gradient gel electrophoresis (DGGE) and single strand conformation polymorphism (SSCP) were used to screen different clones and to investigate community dynamics and succession in biofilms and planktonic cells. The community profiles derived from SSCP analysis indicate a stable microbial community over time in both the biofilms and in the planktonic population of oil and water phase, but with differences between the two sample matrices.

Keywords: Biofouling, SSCP, DGGE, diversity, community stability, lubrication oil, bacteria, fungi

Biological detoxification of different hemicellulosic hydrolyzates using *Issatchenka occidentalis* CCTCC M 206097 yeast

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Lignocellulosic materials represent the largest carbon source and therefore energy on the Earth. These include various agro-industrial residues such as straw, bagasse, bark and chips. The high xylose content in the hemicellulose fraction of these materials has attracted the researchers’ attention for the effective use of this fraction in bioconversion processes. The use of hemicellulose hydrolyzates as a means of crops for conversion of xylose is difficult due the presence of compounds that are inhibitors of microbial metabolism, which are originated by the acid hydrolysis of biomass. Among these inhibitors include the aliphatic acids, furans and phenolic compounds. This study evaluates the detoxification of hemicellulose hydrolyzates of different biomass plants using *Issatchenka occidentalis* CCTCC M 206097 yeast. The hemicellulose hydrolyzates of sugarcane bagasse, sugarcane straw, coffee husk and corn fiber were obtained by dilute acid hydrolysis and concentrated under vacuum to obtain a xylose concentration about 50 g/L. The yeasts inocula were obtained by previous cell growth in semi-defined medium (containing glucose as carbon source). Biodegradability experiments were conducted in 125 mL Erlenmeyer flasks containing 50 mL of hydrolysate with initial pH 5.50. All media were supplemented with 5.0 g/L (NH₄)₂SO₄, 1.0 g/L KH₂PO₄, 0.5 g/L MgSO₄·7H₂O, 0.1 g/L CaCl₂, 0.1 g/L NaCl, 0.2 g/L yeast extract, and 2.0 g/L urea and incubated at 200 rpm and 30 °C for 36 h. Xylose and other compounds were quantified by HPLC. It was observed reduction of xylose (2.45%) and acetate (42.96%) only when coffee husk and sugarcane straw hydrolyzate, respectively, was detoxified. Varied percentages of inhibitory compounds removal was observed in accordance with their initial concentrations in the different hydrolysates. The highest removal of furfural (88.90%) and 5-hydroxymethylfurfural (54.05%) were observed in corn fiber and sugarcane straw hydrolysates, respectively. The results of this study demonstrate the potential use of *Issatchenka occidentalis* CCTCC M 206097 yeast for detoxification of various hemicellulose hydrolyzates, this has demonstrated ability to metabolize furfural, 5- hydroxymethylfurfural and other inhibitory compounds present in hemicellulose hydrolysates.

Keywords: hydrolysate, biodetoxification
Biological polyhydroxybutyrate production from waste glycerol at bench scale

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Polyhydroxyalkanoates (PHAs) are polymers that can be accumulated as intracellular energy and carbon reserve materials by various microbial strains under unbalanced growth conditions. The chemical composition of polyhydroxyalkanoates (PHAs) and thus their properties can vary immensely according to the producing organism and to medium composition. The most common type of PHA is the homopolymer poly(3-hydroxybutyrate); P(3HB); however, copolymers such as poly(3-hydroxybutyrate-co-4-hydroxybutyrate) (P(3HB-co-4HB)) can also be produced if adequate precursors are present in the growth medium. This type of copolymers were found to have adequate properties for medical and pharmaceutical applications [1]. Being biodegradable and produced from renewable sources, (PHAs) will advantageously replace many petroleum-derived plastics if they can be obtained at competitive prices from improved bioprocesses. Economical evaluation studies led to the conclusion that almost 48 % of the total production costs was ascribed to the raw materials, in which the carbon source for growth and polymer accumulation could account for 70 % to 80 % of the total cost [2]. The carbon source is therefore a key factor for PHAs industrial production. To reduce the production cost related with the raw materials, the development of cultivation processes with a high productivity based on waste glycerol (GRP), a by-product of the biodiesel industry, is being addressed in our group.

A Cupriavidus necator strain was chosen to produce both P(3HB) and P(3HB-co-4HB) from waste glycerol and from commercial glycerol as control substrate. For P(3HB-co-4HB) biosynthesis, gamma-butyrolactone (GBL) was used as the precursor. Fed-batch high cell density cultures were developed in a 2 L bench-scale mechanically stirred reactor (STR) and in a 3 L air-lift reactor (ALR) operating at airflow from 1 to 3vvm. Cultures are carried out with control of dissolved oxygen, pH and temperature. In both cases, polymer accumulation was triggered by nitrogen limitation [3]. The cultivation processes are being optimised, in terms of PHA production and productivity. The performance of both bioreactor types will be compared.

The thermal and mechanical properties of the obtained copolymers are determined at the Universities of Liège and Strasbourg. It was verified that, even at low percent incorporation of 4HB monomers in the polyester chain, the properties of the polymers differ from those of the homopolymer P(3HB), namely the crystallinity and melting temperatures.

Keywords Polyhydroxyalkanoates, C. necator, P(3HB-co-4HB), high cell density cultures.


Acknowledgements

This work is being financed by the EU Integrated Project BIOPRODUCTION (NMP2-CT-2007-026515). Catarina D. Almeida and João M. Cavaleiro are supported by fellowships from FCT, Portugal (SFRH/BPD/26678/2006 and SFRH/BD/45266/2008).

Biosynthesis and Biocompatibility of polyhydroxyalkanoates by Cupriavidus necator A-04 and recombinant Escherichia coli

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Homopolymer poly(3-hydroxybutyrate), P(3HB), and its copolymer poly(3-hydroxybutyrate-co-4-hydroxybutyrate), P(3HB-co-4HB), consisting of 9, 5, 24, 38 or 64 mol% of 4HB monomer, were produced in reasonably high content by Cupriavidus necator strain A-04, which was isolated from soil in Thailand. The carbon sources included fructose and 1,4-butanediol as well as sugars made locally in Thailand: refined sugarcane, brown sugarcane, neck sugar, toddy palm sugar and coconut palm sugar. The highest PHB content achieved was 18% (w/w) and the biomass was 3.1 g/l when 20 g/l of brown sugarcane was used. Next, intermittent feeding of carbon sources was performed by adding 4 g/l of the carbon source 5 times, at 12 h intervals. Using this method, the PHB content increased to 40% (w/w).

Therefore, it is possible to use local renewable resources in Thailand for PHA production. Currently, the optimisation of this process using other newly screened bacteria from soil in Thailand is under investigation. Next, bioprocess optimization of P(3HB-co-4HB) production in fed-batch cultivation was achieved and P(3HB-co-4HB)s with 0, 5, 24, 38 or 64 mol% of 4HB content were extracted, purified and used to prepare plastic films. The biocompatibility were tested in comparison with commercially available P(3HB), polystyrene (PS) and polyvinylchloride (PVC). The in vitro biocompatibility was assessed using three different mammalian cell types: L929 (mouse fibroblast cells), Saos-2 (human osteosarcoma cells) and human dermal fibroblast. The results demonstrated that our produced PHB and P(3HB-co-4HB)s were non-cytotoxic to all three cell lines and possess the potential for use as biomaterials in mammalian tissue culture. Aside from the production of PHAs by C. necator strain A-04, we undertook cloning and identification of the PHA biosynthesis genes by PCR and gene walking techniques. Three major open reading frames (ORFs) were obtained and consisted of phaC (1,182 bp), phaA (1,882 bp) and phaB (741 bp) genes encoding for PHA synthase, β-ketothiolase, and acetoacetly-CoA reductase, respectively. The recombinant Escherichia coli strain TOP10 harboring phaCAB genes of C. necator strain A-04 was constructed and expressed under the control of the arabinose-inducible araBAD promoter (pBAD/TOP08 ThioFusion383) for soluble and regulated expression in E.coli. The preliminary study showed that expression of these genes in E.coli results in a significant level (68% dry cell weight) of PHB production at 12 h of cultivation comparing with C. necator strain A-04 accumulated 78% PHB at 60 h. This result indicated the ability of the recombinant E. coli to express the phaCAB genes and possessed the rapid production of PHB.

Keywords Polyhydroxyalkanoates; Biocompatibility; Biosynthesis
Bioavailability of microorganisms increased the accumulation of the end products in this case, lipids, is of great importance for their industrial use. Only some microorganisms do have such ability, in the first place yeasts. The yeasts *Rhodotorula gracilis* are of particular interest because they can develop on the carbohydrate substrate effectively and synthesize a large amount of the lipids (to 70% of dry biomass).

We have analyzed the strain *Rhodotorula gracilis* 17k obtained as a result of screening. This kind yeast’s lipogenesis depends on cultivation conditions. The Carbon to Nitrogen ratio of the growth medium influences the biosynthesis shift towards lipids or proteins formation. For the carbohydrate nutrition the optimal N/C ratio is 1:40.

To provide directed biosynthesis of the lipids an easily assimilated nitrogen source was used in growth medium. Besides that, K₂HPO₄, MgSO₄·7H₂O, and CaCl₂ were added to the medium. The yeasts *Rhodotorula gracilis* 17k were grown during 7 days in the aeration conditions. Being cultivated in laboratory conditions this strain accumulated to 65% of lipids in the biomass. The maximal accumulation of 65-70% of dry matter was registered on the 4th day.

The influence of main elements of the growth medium (trace elements) tells upon the growth intensity of the yeasts and speed of the carbon source utilization, what, in its turn, has an effect on the quantity of the accumulated lipids. Other growth parameters such as pH, temperature and aeration, also have a considerable effect on the fractional composition of the synthesized lipids. While regulating the temperature, different ratios of saturated to unsaturated fatty acids can be created.

The lipids (microbial fat) were extracted from yeasts biomass by ether extraction. By physical and chemical properties the isolated lipids are close to vegetable oils used for technical purposes in different fields of industry. The standard processing of oils obtained from agricultural cultures for diesel mixture can be used also for the microbial fat (biofuel) and for further use of it to obtain biofuel.

**Keywords:** lipids, yeasts, biofuel

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**Changes in the stability and catalysis of fungal enzymes produced in submerged and solid-state fermentation: the case of tannase**

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³ The stability and catalysis of fungal enzymes produced in submerged and solid-state fermentation (SSF), where fungal cells are suspended in a large volume of water that is stirred and aerated using mechanical devices. In such systems, chemical composition of the culture medium is nearly spatially homogeneous because the mixing rate is faster than the reaction rate. But there is another alternative technique for enzyme production called solid-state fermentation (SSF), where the fungal cells are grown on the solid surface of porous materials (biodegradable or not).

In this system, a large fraction of the fermentation mash is occupied by interstitial air and the biomass in in contact with a high level of humidity adsorbed to the mash surface or absorbed into the solid particles supporting the fungal cells. Evidently, the culture conditions affect the physiology of the fungal cells changing some properties of the enzymes produced. In this study, the changes of stability and catalysis of the fungal tannase produced in SmF and SSF were evaluated.

The enzymes produced on both culture systems were purified by isoelectric focusing, ionic exchange and gel filtration chromatography. The specific activity was 5.5 times higher on SSF than SmF.

Tannases produced under SmF and SSF had an isoelectric point of 4.6 and 3.3, respectively. The optimal pH values for both enzymes were found at 6.7 and the pH stability of SSF and SmF tannases were at 6 and 5.8, respectively. Optimal temperatures were from 50 °C for SmF tannase and 60°C for SSF tannase, and both enzymes showed tolerance to high temperature (until 70°C). SSF tannase exhibited a major specificity for methyl gallate (7.8x10⁻⁴M) and SmF tannase for tannic acid (4.9x10⁻⁴M). Several metals had a strong inhibitory effect on SSF tannase. Fe²⁺ and Ca²⁺ showed inhibition on SmF tannase (63% and 7%, respectively). SDS-PAGE analysis as well as gel localization studies of both SSF and SmF purified tannases revealed a single band with molecular weight of 102 kDa and 105 kDa, respectively. Deglycosilation of SSF tannase (reduced) provoked the migration of a second band of 78kDa.

**Keywords** Tannase, stability, catalysis, submerged fermentation, solid-state fermentation
Clavulanic Acid Degradation in an Aqueous Two Phase System

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Polyethylene glycol (PEG) is a stable molecule with low toxicity available in several molecular weights whose properties allow for their use in biomedical and biotechnological applications. One of the most important characteristics of PEG is its formation of an aqueous two-phase system in solution with another polymer or salt. Due to its easy and reliable increase in scale, and the possibility of developing a continuous process of extraction and rapid selective separation, the aqueous two-phase system composed of PEG and potassium phosphate has been studied as an alternative for the purification of the antibiotic beta-lactamase inhibitor, clavulanic acid. However, clavulanic acid has no strongly hydrophobic group and presents high degradation rates in basic and acid regions. These factors lead to low-yield extraction and purification processes compared to those of other β-lactam compounds. The growing interest in the use of this extraction and purification technique has required improvements in the aqueous two phase system for extracting and purifying antibiotics. Aiming to contribute to the studies on clavulanic acid, the authors characterized the degradation of clavulanic acid in aqueous two-phase systems composed of PEG with molecular masses of 400, 600, 1000, 4000 and 6000, with pH of 5.4, 7.0 and 8.0, at a temperature of 20°C. The solutions containing clavulanic acid were obtained from the pharmaceutical product Clavulin®, imported by GlaxoSmithKline Brasil Ltda, and Streptomyces clavuligerus culture broths. After fermentation, the culture broth was pretreated by ultrafiltration through membranes with 3kDa pore size and the concentration of clavulanic acid, in all the aforementioned conditions, was determined by spectrophotometry. The results indicate that the highest stability of clavulanic acid in the presence of PEG occurs at pH 5.4. They also show that PEG with the lowest molecular mass undergoes the highest degradation rate at the same pH. The degradation rate of clavulanic acid in the bottom phase is approximately between 3 to 20 times higher than in the top phase, i.e., the phase where PEG is located.

Keywords: clavulanic acid, degradation, aqueous two-phase system

Cloning and expression of an aspartate aminotransferase from Xanthomonas oryzae pv. oryzae

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A gene encoding an aspartate aminotransferase was isolated from Xanthomonas oryzae pv. oryzae. The cloned gene was inserted into a pGEM-Teasy, a cloning vector. The gene was ligated with a pET-21(a) vector containing His-tag and expressed in E.coli BL21(DE3). Purification of the enzyme with Ni-NTA resin resulted in one-band by analysis using SDS-PAGE. The purified enzyme showed a molecular weight of 43 kDa, as expected. The enzyme was the most active toward L-aspartate as an amino donor, showing that the purified enzyme is one of aspartate aminotransferase exist in X.oryzae pv. oryzae. The aspartate aminotransferase also showed an activity toward L-leucine and L-cysteine, but to a lesser extent. Optimal activity of the enzyme was observed at around pH 7.5. The stability was much higher at alkaline pH rather than acidic pH values. The optimal temperature ranged from 35 to 40°C and the residual activity after heat treatment at 55°C for 20 min was 78% of the initial activity. The enzyme was considerably activated by the presence of manganese ion, showing about 157% of initial activity at 1.0 mM addition.

Keywords: aspartate aminotransferase, Xanthomonas oryzae
Cultivation of *Salmonella enterica* Typhimurium in bioreactor and development of flagellin purification process

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Flagellin is the main protein present in bacterial flagellum with some 20,000 sub-units (each 50 kDa). Its role as an adjuvant vaccine has been intensively studied and currently there is evidence to be an immunomodulator in situations of autoimmune diseases. The purpose of this work is to establish a production process and native flagellin purification. Cultivation was carried out in bioreactor. Flagellin was extracted from the cell and the released flagellin to the supernatant was purified by using tangential ultrafiltration in membrane with cut-off 700 kDa and 300 kDa. Flagellin recovery in the concentrated fraction of 700 kDa was 33%, and ~17% of the flagellin was detected in the UF700kDa. Volumetric production obtained in this process was 101.14 mg/l culture that means higher than the traditional method (10 mg/l culture). The results indicate a perspective to establish a production process and flagellin purification process easily to scale-up.

**Keywords:** Salmonella, flagellin, bioreactor, purification, tangencial ultrafiltração.

Degradation assay of lignocellulosic compounds in combination with polyurethane resin by CECT fungi

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Environmental pollution by plastic wastes has become a serious issue and polyester polyurethane resins (PUR) had attracted attention because of its biodegradability. There are many reports on the degradation of lignocellulosic wastes and polyester PUR by microorganisms, especially by fungi. Maderon® is a polymer manufacture with 25% powdered almond shell waste and 75% of polyurethane resin (PUR). For the degradation assay of Maderon, four strains of fungi from CECT (Aspergillus flavus, Trichoderma atroviride, Phanerochaete chrysosporium and Penicillium simplicissimum) were tested. Fungi were grown on minimal broth with 1% (w/v) of residue powder for 72 h. Later, the broth filtered was used to determinate: laccase, Mn-peroxidase, ligninase, aryl alcohol oxidase and cellulase enzymatic activities. Also, the strains were incubated on minimal agar with 1% Maderon plates containing, previously weighed pieces, of residue. Incubation was carrying out at 28º C during 15 days. Then, lost of weight was determined. The plates were incubated with the four strains individually and in multiple mixed cultures. Five replicates were made for each assay.

Results show lower lignolytic enzymatic activities in CECT strains in relation to strains used on industrial applications for ligninolytic wastes degradation. In the pieces degradation, *P. chrysosporium* produces the principal weight loss (1.5%) in simple culture. Result from multiple mixed culture amount the 3.2% of weight lost. In conclusion, the CECT strains are not effective for residue degradation. Several assays with fungi used on industrial application are indispensable to resolve lignocellulosic and PUR recalcitrant compounds biodegradation.

**Keywords:** Maderon®, fungi, lignocellulosis, laccase, Mn-peroxidase, ligninase, aryl alcohol oxidase, cellulase.
Detection of PKS genes from a Brazilian sugarcane endophytic Aspergillus sp.

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Secondary metabolites are a group of low mass molecules synthesized by microorganisms that do not participate on cell growth neither on the constitution of cell components. In spite of the little knowledge of the role of these secondary metabolites in the biology of their producers, biotechnology and pharmaceutical industries have used them in a number of applications such as antibiotics, immune modifiers, and antitumor agents.

Polyketides are a vast and important class of secondary metabolites that show diversity not only in structures but also in functions and applications. Nowadays, the search for new molecules candidates for turn into new chemistry entities has become a prime necessity. Recently, endophytic microorganisms have called attention and contributed with the production of new compounds.

An Aspergillus sp. endophytic fungus has been isolated from sugarcane leaf and assessed for the presence polyketide compounds by PCR approaches, looking for conserved domains into the polyketide synthase (PKS) genes. Phylogenetic analysis of the ITS 1-5.8- ITS2 region confirmed that this isolate belongs to the order of the Eurotiales with a high genetic identity with fungi of Aspergillus genus. PCR amplicons of the ketosynthase (KS) region of the PKS gene showed similarity with sequences of a putative WA type ketosynthase of Penicillium sp. ZH01 (genebank code ABQ55530.1) and a putative hybrid PKS/NRPS enzyme of Aspergillus flavus NRRL3357 (genebank code EED49862.1), with 94% and 99% identity respectively. C methyltransferase region (Cmet) amplicon showed homology similarity with a sequence of a hypothetical Penicillium chrysogenum polyketide synthase Wisconsin 54-1255 (genebank code CAPI9398.1) with 59% of identity. Phylogenetic analysis of KS domains showed that the translated amino acid sequences grouped with the clade II of reduced polyketides, especially in the group of hybrid PKS/NRPS, and in the clade I of non reduced polyketides involved in the synthesis of non-melanin pigments and aflatoxins. The Cmet analysis showed that the amino acid sequence grouped with the clade II of reduced polyketides.

In order to detect polyketide compounds from this isolate, organic extracts were analyzed using chromatographic techniques (TLC, HPLC) and nuclear magnetic resonance (NMR), showing the production of a possible statin compound. This extract also demonstrated biological activity against Bacillus subtilis and also against the statin sensitive strains of Candida albicans and Mucor sp.

Keywords: Endophytic, Aspergillus sp., Polyketide synthase (PKS), Ketosynthase (KS), C methyltransferase (Cmet), statins.

E. coli under pressure – stress monitoring in large scale protein production

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It is known that misincorporation of modified amino acids occurs during the overexpression of recombinant proteins under high cell density fermentation in E. coli. Different kinds of stress negatively influence growth and, therefore, protein expression (1). At non-predictable occasions, modified instead of correct amino acids are incorporated (2) into nascent proteins (Fig. 1). The ratio of misincorporated amino acids in therapeutical proteins should be as low as possible. Since all undesired protein by-products need to be considered in clinical studies and require extensive analytical characterization to obtain a biologics license application. Available methods to monitor the misincorporation are either inexact or not sufficient for every protein.

Our approach includes antibody-based detection of wrong built protein during and after the microbial production process. With high specificity and affinity properties antibodies are able to produce tight protein-protein interactions leading to antibody-bound target protein. We selected binding domains for regions containing non-coding amino acids from a fully synthetic camelid VHH-antibody library by directed competitive phage display. Target specificity is only encoded within a single peptide chain since the recombinant antibodies lack any light chain resulting in better expression and stability (3). These antibodies can be used for the detection of undesired byproducts during fermentation processes and several production relevant purification steps in industrial biotechnology.

Keywords: protein production; misfolding; camelid antibodies; fermentation monitoring;

References
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Effect of coating materials upon organic acid production by immobilized B. animalis subsp. lactis Bb12, in both batch and continuous cultures

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Bifidobacterium animalis subsp. lactis Bb12, following previous immobilization in gellan-xanthan and κ-carageenan-locust bean gum, was used to ferment skim milk-based media in uncontrolled- and controlled-pH chemostat. Lactic, acetic, formic and succinic acids were thus monitored for 24 h and 10 d, during batch and continuous culture, respectively. Gellan-xanthan-entrapped cells produced more formic, succinic and acetic acids than their κ-carageenan-locust bean gum counterparts. Under chemostat culture, the mole ratio of acetic to lactic acid, as well as the rate of production of formic and succinic acids increased gradually after 4 d; the coating materials appeared not to play a significant role upon such changes. The observed variations in organic acid production are relevant because of their effect on the organoleptic features of the final fermented dairy product.

Keywords: Bifidobacterium; Immobilization; Medium

Effect of oxidized cellulose on probiotic bacteria

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Polysaccharides and oligosaccharides may support growths of human colonic bacteria, some of them has inhibitory effect. Positive and negative effects of inulin, oligofructose and acid form of oxidized cellulose were tested by dilute method on selected probiotic bacteria. These samples were tested in 1, 2 and 3 % concentrations on Bifidobacterium bifidum, Enterococcus faecalis, Lactobacillus lactis and Lactococcus spp. bacteria. Positive effects were achieved with inulin and oligofructose, oxidized cellulose influenced positively mainly in lowest tested concentration. Higher concentrations proved inhibiting effects.

Effect of acid form of oxidized cellulose was tested by dilute method on selected probiotic bacteria - Enterococcus faecalis, Lactobacillus lactis and Lactococcus spp - with different values of pH, to simulate an environment of intestinal tract from stomach to colon. Effect of oxidized cellulose was determined with pH 1,4; 5,5; 6; 7 and 7,5 and compared with a blank experiment. No growth was noticed with pH 1,4. The growth of bacteria with other tested pH was lowered by about 1 - 2 levels compared to the blank experiment. The most optimal environment for the growth of microorganisms in the presence of oxidized cellulose was the one with pH 7.

The results indicate that oxidized cellulose could be used (in lower concentrations) for preparation of dietary supplements for body-weight reduction.

Acknowledgement

This study was supported by the Ministry of Education, Youth and Sports of the Czech Republic. Project No. 0021627502 and by GACR No. 203/08/1536, as well as project No. 2A-1FP/073.

Keywords probiotic bacteria, oxidized cellulose, inulin, oligofructose
Effect of pH and Inoculum Percentage on Canthaxanthin Production by Dietzia maris

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Dietzia maris was isolated from our laboratory and was grown in Yeast Extract-Peptone-Glucose medium at 37°C and 180 rpm. The pH of the medium was varied between 1 and 14 and inoculum percentage between 0.5% and 10%. Considerable growth and pigmentation was observed at pH between 5 and 12 and at all inoculum percentage, with the maximum yield at pH 5.5 and 2% inoculum. The pigment was extracted by solvent extraction method using methanol. The pigment extracts were analyzed by scanning the absorbance with a UV-Vis spectrophotometer and the maximum peak was obtained at 483 nm. The pigment was identified as canthaxanthin by HPLC analysis.

Keywords Canthaxanthin; pH; Inoculum Percentage

Effects of static magnetic field in Saccharomyces cerevisiae cultures under aerobic and anaerobic conditions

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Magnetic fields have been studied by many researchers as an agent that provides changes in the metabolism of microorganisms. The effects in biological systems are produced by a relative movement between mobile ions or intracellular free electrons and the magnetic field, which can generate electric fields within a cell. Despite of the interaction mechanism to be yet unclear, is known also that the direct action of magnetism cause changes in the conformational shape of cell proteins, altering its functions.

The purpose of this research was to investigate whether static magnetic field (SMF) could be applied to increasing ethanol yield by S. cerevisiae and verifying the biological effects on growth stimulation or inhibition. The influence of aerobic and anaerobic conditions and the ability of the cells to induce their defensive enzymes were also explored.

Batch fermentations of S. cerevisiae were conducted for 26 h, in YPD medium, in hyperbaric bioreactor (Parr 4563, Parr Instruments, USA) with 5 NdFeB magnets, with an average flux of 120 mT (measured by a PHYWI Teslameter, USA). The magnets were enclosed into a transparent flat polyethylene case with 4.0 x 3.5 cm and 1.3 cm width. All magnets were disposed with the same polarity, so when closed the case every one showed similar north or south poles at each face of the case. As they repelled each one, the magnets maintained the maximal distance between them, in an “X” disposition, with a magnet in the center. Controls were performed in the same manner without magnets. To investigate the effects of gas, aerobic and anaerobic conditions trials were performed under air (1 bar and 5 bar) and nitrogen (1 bar). An increase of the cell dry weight and specific growth rate, under aerobic conditions (5 bar of total air pressure), of approximately 2-fold was obtained compared with the experiments exposed to nitrogen, for both magnetized and non-magnetized cultures. The cellular growth was not stimulated by the application of SMF. In fact, an improvement of 2-fold in specific growth rate was obtained in the control group, for aerobic and anaerobic conditions.

An increase of approximately 3-fold in biomass yield was obtained in aerobic cultures compared with anaerobic. The biomass yield of exposed group to SMF was similar to non-exposed one under 5 bar of air pressure and in nitrogen cultures. On the other hand, a stimulation of ethanol production was obtained in magnetized cultures relatively to those of the controls, for both aerobic and anaerobic conditions.

Generally, antioxidant enzymes were induced by total air. An increase of the SOD specific activity in magnetized cultures was obtained compared with the control experiments. Also, the SMF exposed cultures reached the highest values of the catalase specific activity. However, SMF exposure led to a decrease in the glutathione reductase activity.

The marker malondialdehyde (MDA) is certainly the most widely used to assess the lipid peroxidation processes. The use of anaerobic conditions resulted in a reduction of MDA levels, for both exposed and nonexposed groups. Generally, the MDA concentration was similar for both magnetized and nonmagnetized cultures. This work shows that magnetic fields can be applied as a controlling factor of S. cerevisiae fermentations for ethanol or enzymes production.

Keywords: static magnetic field (SMF), Saccharomyces cerevisiae, aerobic and anaerobic conditions, ethanol.
Estimation of Colony Forming Units in 3 minutes for individual cell types in a mixed culture using Methylene Blue Dye Reduction Test

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Determination of cell viability is an important step in designing, operating and controlling fermentation processes. It is more relevant in processes involving mixed cultures, where multiple cell types contribute to the total growth. The motivation for the current study is to develop a methodology to estimate viability counts for the individual cell types in a mixed culture. Further, the methodology should indicate the presence of a contaminant in short time periods since in the agar plate methods used frequently it takes about 24-30 hrs. We present a methodology based on the rate of Methylene blue (MB) reduction to evaluate the total count of viable cells. The standard curve relating the slope of MB reduction and CFU of the individual cells could be used to measure the viability of each cell type in the mixed culture. The slope of MB reduction could also be used to obtain the growth rate of individual cell types in a mixed culture and that of the total cell count. These measurements where achieved in less than 6 minutes during the growth of the cells. Evaluating the cell viability of individual cell types in a mixed culture is tedious, difficult and time consuming. The Methylene Blue Dye Reduction Test (MBRT) presented here is capable of quickly estimating colony forming units of individual cell types in a mixed culture. The method was used to dynamically determine the presence of a contaminant during fermentation. The protocol developed here can be adapted to applications in processes involving mixed cultures.

Nomenclature:
MBRT: Methylene Blue Dye Reduction Test,
MB: Methylene Blue.

Keywords: Colony forming units (CFU), viability, Methylene blue dye, Dye decoloration, Cell membrane enzymes, Escherichia coli, and Bacillus subtilis.

Ethanol fermentation of Carob pods extract by “Zymomonas mobilis” bacteria

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Depletion of fossil fuel reservoirs, world environmental problems as well as economical and practical pressures has motivated many researches in the field of production of bioethanol as a renewable and environmental friendly fuel based on fermentation of carbohydrate containing agricultural crops. One of the high carbohydrate containing agricultural crops is “carob pod” which its Latin name is “Ceratonia siliqua”. In spite of high amount of sugars contained in carob pods namely Sucrose, Glucose, and Fructose; only a few research works has been published on ethanol production of this potentially suitable crop by “Saccharomyces cerevisiae”. Zymomonas mobilis is an alternative microorganism to S. cerevisiae which its useable sugars are restricted to Sucrose, Glucose, and Fructose. These are the same sugars as present in carob pods. Z. mobilis is a gram negative bacteria and superior to S. cerevisiae because of; its higher sugar uptake and ethanol yield, its lower biomass production, its higher ethanol tolerance and its amenability to genetic manipulations. Also Z. mobilis cultures grow anaerobically and unlike yeasts do not require the controlled addition of oxygen to maintain viability at high cell concentration. This study was designed not only to evaluate the potential of carob pods extract for ethanol production but also to optimize the carob pods extract fermentation conditions by Plackett-Burman and Response Surface Methodology in order to reach the maximum gram ethanol production per gram initial sugar contained in carob pods extract culture media as well as a comparison was made between the fermentation results of acid hydrolyzed and non hydrolyzed carob pods extract. Effects of inoculum amount, initial sugar introduced via carob pods extract, amount of peptone, yeast extract and time on response factor (gram ethanol produced per gram initial sugar) was studied at initial pH 5.17, Temperature 30℃, rpm 80 in shaken-flask. The best condition for maximum response were (as gram per total 50 ml culture media) inoculums bacteria 0.017, sugar 5.78, peptone 0.42, yeast extract 0.42 and time 48 hours; achieving 0.335 gram ethanol produced per gram initial sugar in culture media. Theoretical ethanol yield was 92.4685%. Secondly fermentation of acid hydrolyzed carob pods extract resulted to lower theoretical ethanol yield (88.3289%) and higher fermentation efficiency (78.1166%) in comparison with fermentation of non hydrolyzed form 92.4685% and 72.2907% respectively. The response in hydrolyzed treatment was higher than non hydrolyzed treatment by 3.2162%.

Keywords: Ethanol, Zymomonas mobilis; Carob pod; Fermentation; Response Surface Methodology; Optimization
Evaluation of different microbial expression systems for therapeutic peptide production

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Peptide and protein drugs, classified as biopharmaceuticals or biologics, exhibit an increasing share of the pharmaceutical market. Biopharmaceuticals include drugs with wide therapeutic application, for certain indications they even represent the only effective therapy – for example treatment of cancer, autoimmune diseases, diabetes, anemia, disorders associated with lack or damage of certain proteins and many others. In the past, therapeutic peptides and proteins were isolated from natural sources but then their production has shifted to new and more advantageous biotechnologies such as recombinant DNA techniques which moreover allow engineering of peptides and proteins to have optimal pharmacological properties.

The aim of our work is to prepare and compare several microbial expression systems for production of therapeutic peptides in high-yields, mainly employing bacteria (Escherichia coli). Further step of our effort is to optimize purification conditions from microbial culture to get the therapeutic peptide in high purity, and finally stabilize the peptide for its therapeutic use in human body and prepare a convenient peptide drug formulation.

Production of biologically active peptides in bacteria Escherichia coli is unfortunately limited because of very low yields. This is caused mainly by rapid intracellular degradation of the recombinant peptides, as well as the difficulty in purification from contaminating proteins and peptides. Several methods leading to increased peptide yields in bacteria E. coli have been tested. One method relies on use of fusion partners (i.e. glutathion-S-transferase (GST), maltose binding protein (MBP), etc.) (described in Fig. 1). By including an appropriate protease recognition sequence, the peptide can be separated from the fusion partner by proteolytic fission (i.e. by enterokinase, factor Xa). Another method involves gene polymerization (described in Fig. 2). Here, the gene of interest is expressed in several copies and peptide-polymer is subsequently cleaved into monomers (i.e. by CNBr).

We have developed several expression systems with the aim of yield enhancement of peptide production in Escherichia coli. Approaches based on use of fusion protein and gene polymerization technique have been shown as acceptable methods for production of therapeutic peptides in E. coli. The highest yields were obtained with gene polymerization approach. Currently we focus on stabilization of the therapeutic peptide in human body by use of a suitable biodegradable polymer.
Fermentation characteristics as criteria for selection of cachaça yeast

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The fermentation characteristics of 24 strains of Saccharomyces cerevisiae and one strain of Candida apicola, C. famata, C. guilliermondii, Hanseniospora occidentalis, Pichia subpelicullosa and Schizosaccharomyces pombe were evaluated for the production of cachaça. They were isolated from small cachaça distilleries (27), industrial cachaça distilleries (2) and one sugarcane alcohol distillery. The yeasts showed significant differences in ethanol yield, substrate conversion, efficiency, conversion factors of substrate into ethanol (Yp/s), cells (Yx/s), organic acids (Yac/s) and glycerol (Yg/s), and maximum specific growth rate (μmax). In general the S. cerevisiae strains showed better fermentation potential, with yields between 83 and 91% and μmax between 0.450 and 0.640 h⁻¹, several of them being comparable with the high performance yeast used in the industrial production of ethanol, which was adopted as a reference. The non-Saccharomyces strains showed high efficiency, very low ethanol yield and very high Yac/s and Yg/s values, except Pichia subpellicullosa, which behaved very similar to the S. cerevisiae strains. Hierarchical Cluster Analysis and Principal Component Analysis showed the fermentation yield (or substrate conversion) as being the variable which most contributed to the separation of the strains into different groups.

Keywords: alcoholic fermentation, cachaça, Saccharomyces cerevisiae, yeast

Acknowledgements: We thank Fundação de Amparo à Pesquisa de Minas Gerais (FAPEMIG) for financial support

Fermentation characteristics of Saccharomyces cerevisiae strains to produce banana’s brandy

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Four Saccharomyces cerevisiae yeast strains, as well as the wet, pressed commercial yeast (for baking bread) were evaluated with respect to their fermentation characteristics, production of methanol and higher alcohols viewing the production of distilled spirits from banana. All the strains used were isolated from small “cachaça” distilleries, with the exception of the commercial yeast. The yeasts showed significant differences in the fermentation parameters such as the yield of ethanol and efficiency of conversion total reducing sugars (TRS) to ethanol. The wet, pressed commercial yeast and UNICAMP V1 strain were prominent in presenting the best results for the yield in ethanol and efficiency. There were no significant differences between the wet pressed yeast and the UNICAMP V1 strain with respect to these parameters. The UFMG A1240 strain presented the lowest yield of ethanol (69.16%) being non suitable for production of banana’s brandy. The methanol’s concentration of all strains evaluated did not change significantly (P ≤ 0.05), with except UFMG A1007 strain, which produced significantly higher levels than the others (0.19 mL/100 mL anhydrous alcohol). The higher alcohols produced varied significantly among the strains and the lowest concentrations were the UFMG A905 and UFMG A1240 strains (30.04 and 48.69 g/100 mL of anhydrous alcohol). The UNICAMP V1 strain produced low levels of higher alcohols (82.26 g/100 mL of anhydrous alcohol) and showed good yield of ethanol (83.07%), making it more suitable for the production of banana’s brandy.

Keywords: yeast, Saccharomyces cerevisiae, banana brandy, alcoholic fermentation.

Acknowledgements: This work was funded by Fundação de Amparo à Pesquisa de Minas Gerais (FAPEMIG)
From *in silico* to *in vitro*: Modelling and production of *Trichoderma reesei* endoglucanase 1 mutants in *Pichia pastoris* for textile biofinishing

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Wide range of application of enzymes allowed their use in many textile processes such as biopolishing, biostoning, desizing and bleaching. Among all of the enzymes cellulases are being used increasingly for a variety of industrial purposes and consequently lots of effort has been put into their cloning and expression as well as their study by site-directed mutagenesis. Cellulases are the major cellulolytic enzymes that degrade cellulose. Nearly all of the cellulases can work at neutral and acidic pH and wide temperature ranges. Some industrial applications require heat stable enzymes to eliminate side reactions. Cellulases are used in textile biofinishing for the removal of the fuzz on the surface of the fabric in an attempt to reduce pilling of the fabrics but they cause a loss in the tensile strength of fabric.

The aim of this study is to improve the enzymatic action of a major cellulase, namely endoglucanase 1 (EG1) from *Trichoderma reesei* for viscose biofinishing by the introduction of ten amino acid long loop mutations at various locations, and by analyzing the impact of the proposed mutations on the protein stability via molecular mechanics (MM) and molecular dynamics (MD) simulations.

Molecular Mechanics (MM) and Molecular dynamics (MD) were performed on EG1. Weak points that cause unfolding of the enzyme were found by MM studies performed at 400-450 and 500 K. Key functional residues were found by MM studies. Moreover, functional, structural and sequence motifs were found by using machine learning methods. The key functional positions were mutated using stability motifs found and the impact of these changes on thermal stability of the enzyme was analyzed using MD studies. Best loop mutation predicted *in silico* was introduced to endoglucanase 1 via site directed mutagenesis. The loop mutant and the wild type enzymes were both expressed in *Pichia pastoris* and wild type and mutant enzymes were characterized and their activities against soluble and insoluble substrates such as CMC, 4-MUC and their effects on the properties of viscose (such as pilling, bursting strength) were determined. Activity and the thermal stability of the loop mutant was found to be comparable to the wild type. The mutant enzyme was found to be capable of alleviating the problem of fuzz removal from viscose fabric without disrupting the fabric strength.

**Keywords** cellulase; endoglucanase; modelling; site directed mutagenesis; viscose biofinishing

References


Scale-up of biosurfactant production process by *Candida lipolytica* UC P 0988

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Scale-up of biosurfactant production process by *Candida lipolytica* UC P 0988 from the initial 0.1-L shake-flask scale to the 5.0-L lab-scale was successfully carried out. Initially, the biosurfactant production medium was optimized in Erlenmeyer-flask scale. After, the effects of agitation rate and process temperature on the biomass concentration and the emulsification activities for emulsions water-in-hexadecane, water-in-corn oil and water-in-canola oil were studied in lab-scale using design of experiments (DOE) and surface response methodology (RSM). The greater biomass concentration (27.78 g/L) and the greater emulsification activities for emulsions water-in-hexadecane (5.44 UEA), water-in-corn oil (5.92 UEA) and water-in-canola oil (5.8 UEA) in biosurfactant production process were found to be associated with high agitation rates (300 rpm) and low temperature (28º C). However, high biomass concentration (16.103 g/L) and high emulsification activities for emulsions water-in-hexadecane (5.45 UEA), water-in-corn oil (6.1UEA) and water-in-canola oil (4.624 UEA) in biosurfactant production process were also reached with low agitation rates (150 rpm) and low temperature (28º C). In this last condition, the cell-free filtrate containing the surfactant produced by *Candida lipolytica*, presented surface tension of 33 mN/m.

**Keywords** scale-up, factorial design, response surface methodology, biosurfactant, bioemulsifier, *Candida lipolytica*.
Hydrogen Production from Glycerol using Halophilic Fermentative Bacteria

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Halophiles are microorganisms that require high salt concentration in their living environment for growth and survival. Some halophilic algae cope with hypersaline environments by the production and accumulation of organic solutes e.g. glycerol. Glycerol plays an important role in hypersaline environments. Few halophilic fermentative bacteria including Halanaerobium saccharolyticum are capable for utilizing glycerol as substrate in their metabolism. Here we present a study on end metabolite production in the glycerol metabolism of Halanaerobium saccharolyticum subspecies saccharolyticum and senegalensis which are halophilic anaerobic bacteria capable for glycerol fermentation and hydrogen production. Studies were conducted as batch experiments and metabolites were analysed as end-point measurements. The main metabolites of glycerol fermentation of both H. saccharolyticum strains are H2, CO2, and acetate. Optimal conditions for H2 production were defined. Halophilic glycerol fermentation was compared with glucose fermentation. The highest hydrogen production yield of H. saccharolyticum was 0.52 mol/mol and for H. saccharolyticum subsp. senegalensis 1.13 mol/mol, which are 52 % and 113 %, respectively, of the theoretical maximal hydrogen yield of Escherichia coli (1 mol/mol glycerol). Halophilic glycerol metabolism was also compared with the metabolism of known non-halophilic hydrogen producers, E. coli and Clostridium butyricum that are capable for using glycerol as substrate.

Taken together, we have shown that halophilic bacteria are promising candidates for future bioenergy producers. Their habitat provides excellent selection circumstances in big bioprocess conditions where open system without sterilization possibilities is the only choice. We shall show some data also on thermophilic anaerobic halophiles which provide extra selection possibilities. Discussion on metabolic engineering of the hydrogen producing hosts will be conducted.

Keywords: extremophile, halophile, thermophile, anaerobic bacteria, hydrogen, glycerol, fermentation

Hydrolysis of spruce wood and sugarcane bagasse by cellulases and hemicellulases

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The search of renewable energy solutions is a worldwide research topic: Plant biomass is an attractive renewable resource for production of biofuels and materials within the biorefinery concept. Hydrolysis of cellulose and hemicelluloses can be achieved by enzymes. In this study we have focused on spruce wood and bagasse, two raw materials that vary in composition and global distribution. Spruce wood and bagasse consist mainly of lignin, cellulose and hemicelluloses. Galacto-glucomannan is the major hemicellulose component in spruce wood (1) whereas xylan is the major hemicelluloses in bagasse (2).

Research attempts on improving efficiency of cellulose hydrolysis rely on the knowledge of hydrolysis mechanisms. Hydrolysis studies using realistic substrates are needed since the composition and structure of substrate components may well affect the efficiency of certain enzymes. The synergy between cellulases during the hydrolysis of lignocelluloses has been previously studied (3). These findings show that synergy between cellulases varies depending on the substrate used and the enzymes added. Synergy involving hemicellulases has been less studied although a few studies have been published (4). Many hemicellulases are modular and have carbohydrate binding domains (CBDs) that bind to the substrate. For cellulases, binding during hydrolysis might lead to a decrease in hydrolysis rate due to unproductive enzyme binding (5), but if this may be the case for hemicellulases as well is less known.

Our interest is to get knowledge on the role of mannanase and xylanase in the hydrolysis of real substrates. For this aim natural, spruce chips pretreated spruce and sugar cane bagasse were used as substrates. We have chosen commercial Celluclast 1.5L, and Novozyme 188, purified TrMan5A mannanase, XylXIB xylanase and TrCel12A cellulase. We will discuss hydrolysis rate at different loads of cellulases and results of the addition of mannanase in the hydrolysis of spruce and spruce pretreatments. We have also studied the hydrolysis rate declination of TrCel12A during hydrolysis of this complex substrate.

Keywords: mannanase, xylanase, cellulase,cellulose and hemicelluloses hydrolysis, raw material.

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Improving bioethanol production by Saccharomyces cerevisiae strains, using agro-industrial by-products

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Bioethanol is a relevant renewable energy, particularly in transport sector, as it can contribute to reduce the global energy dependence on fossil resources and mitigate CO2 emissions leading to climate change. Since 1980, ethanol has been an established alternative to fossil fuels in Brazil, produced mainly from sugar cane and is the most widely produced biofuel, with over 50 000 millions L in 2007. The use of others single energetic crops, as crop and maize result in an intensive agriculture which can have high environmental costs in a short-term. Nowadays, the challenge is to increase the production efficiency from the feedstock, improving the economy of the bioprocess, becoming it competitive with fossil fuels price.

So-called 2nd generation biofuels use organic waste, mainly from agriculture and lignocellulosic residuals. Our proposal is the development of a sustainable bioethanol production with high efficiency, using industrial by-products, carob pod, a cheap feedstock, rich in sugar.

Fermentations were performed, at laboratory scale, using different Saccharomyces cerevisiae strains and carob extract sugar contents, between 40 g/L and 250 g/L. The best ethanol production was 100g/L, after 60 hours with an ethanol yield of 43 %. Specific growth rate was similar to the already described (Raposo et al, 2009). No growth inhibition was observed due to high phenolic content of the carob extract.

Scaling-up studies are being carried out with the strains that showed higher vigor and higher ethanol productivity. These results may contribute for the economical viability of the bioethanol process from carob extract and it can be a promisory alternative in the near future.

References


Inhibitory action of the toxic compounds present in lignocellulosic hydrolysates on xylose-to-xylitol bioconversion by Candida guilliermondii

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Lignocellulosic materials represent an abundant and inexpensive source of sugars which can be microbiologically converted to industrial products. However, hydrolysis of lignocellulosic materials for sugars recovery always goes together with the formation of by-products that inhibit the fermentation process. Such by-products include acetic acid, phenolic compounds such as syringaldehyde, ferulic acid, p-hydroxybenzoic acid, and vanillic acid, among others. These toxic compounds can stress fermentative organisms to a point beyond which the efficient utilization of sugars is reduced and product formation decreases. The inhibitory concentration of these compounds varies between each microorganism and the knowledge about the maximum tolerable concentration and how to minimize their effects is of great importance to attain efficient fermentative processes. Regarding the industrial products that can be generated from lignocellulose-derived sugars, xylitol has received much attention, since it presents particular physic-chemical properties that can substitute for the treatment of diabetes and erythrocytic glucose-6-phosphate dehydrogenase deficiency. In this study, the inhibitory action of acetic acid, ferulic acid and syringaldehyde on metabolism of Candida guilliermondii yeast during the xylose-to-xylitol bioconversion was evaluated. The concentration values evaluated for each toxic compound were based on values usually found in lignocellulosic hydrolysates.

For a good understanding of the individual effect of each toxic compound, the assays were performed in semi-defined medium containing (g/l): xylose (85.0), glucose (15.0), (NH4)2SO4 (3.0), CaCl2*2H2O (0.1), KH2PO4 (21.7), K2HPO4 (6.9), and 20% (v/v) rice bran extract; supplemented or not with acetic acid (0.8 to 2.6 g/l), ferulic acid (0.2 to 0.6 g/l) or syringaldehyde (0.3 to 0.8 g/l). The experiments were performed in 250-ml Erlenmeyer flasks containing 100 ml of fermentation medium inoculated with 3 g/l cells. The flasks were incubated at 30 ºC, 200 rpm, for 96 h. During the fermentation, samples were withdrawn each 24 h for determination of the xylose, glucose, xylitol, and cells concentration, as well as the pH variations.

The yeast cell growth was affected by all the evaluated acetic acid concentrations, while the xylose consumption and xylitol formation were only affected in the presence of the highest acid concentration (2.6 g/l). In fact, the presence of this acid concentration in the fermentation medium reduced the cell growth, xylose consumption and xylitol production in 30, 13 and 18%, respectively, when compared to the assay without the acid addition. Cell growth was also reduced in the presence of all the evaluated ferulic acid concentrations, being observed a decrease of 30% in the microorganism growth when the fermentation medium was supplemented with the maximum ferulic acid concentration (0.6 g/l). On the other hand, xylose consumption was not affected by the ferulic acid presence in any evaluated concentration, and the xylitol production was only slightly reduced (15%) when in the presence of 0.6 g/l ferulic acid. Syringaldehyde concentrations higher than 0.3 g/l affected cell growth, being observed a reduction of 36% on biomass formation when the fermentation medium was supplemented with 0.5 or 0.8 g/l of this toxic compound. However, the xylose consumption and xylitol formation were not affected by any of the tested concentrations of this compound.

It was thus concluded that acetic acid, ferulic acid, and syringaldehyde are compounds that may affect the Candida guilliermondii metabolism (mainly the cell growth) during the xylose biotransformation to xylitol, being their toxic effect dependent of the concentration present in the medium. Such results are of interest and reveal that it is not necessary a complete removal of toxic compounds from the fermentation medium to obtain an efficient xylose conversion to xylitol by Candida guilliermondii. Acknowledgements: CAPES, FAPESP and CNPq, Brazil.

Keywords xylose; xylitol; Candida guilliermondii; acetic acid; ferulic acid; syringaldehyde; toxic compounds
Investigating the 3-dimensional structure of family 43 glycoside hydrolase (CtGH43), a cellulase from Clostridium thermocellum structure for possible interactions using molecular docking and other bioinformatics tools

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The objective of current study was to computationally simulate the molecular recognition process from the predicted 3-D structure of CGH43 using MODELLER 9-v4 that showed the expected 5 fold β-propeller core structure that is native to GH-4 clan [1,2,3,4]. The aim of the docking study is to predict and rank the structures(s) arising from the association between a given ligand and our target protein (CtGH43). We tried to achieve an optimized conformation for both the protein and ligand and their relative orientation such that the free energy of the overall system is minimized. Initially we tried to find out the possible catalytic or ligand binding site from the predicted 3-D structure of CGH43. The Ramachandran (RC) plot on RAMAPAGE server revealed that CGH43 contains no segments of helix. The RC plot analysis also showed that among 301 residues, 267 (89.3%) were in favoured region, 23 (7.7%) were in allowed region and 9 (3.0%) were in outlier region. The secondary structure of CGH43 showed the regularly spaced intra-chain hydrogen bonding. We were able to confirm the absence of α-helices and the presence of parallel beta sheets and loops from the HB plot. The 3-D structure of CGH43 showed a large site volume for only 3 ligand binding sites out of 10 predicted ligand binding sites as analysed using Q-Site-Finder. Further investigations using Thematics (Theoretical Microscopic Titration Curves) showed only two positive clusters having two or more amino acid residues [5]. The results of the molecular docking of CGH43 will be presented at the conference [6].

Fig. 1. Ligand binding sites analysis of CtGH43.

Fig. 2. 3-D structure of CGH43 developed utilizing MODELLER 9-v4 and visualized by PYMOL.

References

Isolation and characterization of cellulase producing bacteria from pruning tree compost and soil

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Cellulose is the major component of plant biomass, which is a renewable and abundant resource with potential for bioconversion to value-added products. Cellulose is a homopolysaccharide composed of glucose units, linked by β-(1→4)-glycosidic bonds. To make cellulose to economically competitive, efficient enzymatic processes that hydrolyze the cellulose into fermentable sugars are needed.

Cellobiohydrolases (EC 3.2.1.5) are enzymes that cleave cellobiose to glucose monomers. Effective hydrolysis of cellulose requires these enzymes to break down the cellulose into fermentable sugars. Therefore, a greater understanding of thermostable cellulases could potentially lead to new and useful applications in industry.

In order to obtain novel thermophilic cellulase producing bacteria, we successfully isolated moderately thermophilic bacteria degrading cellulose from pruning tree compost and soil. The sample was suspended in saline, and the supernatant was inoculated into modified Brock’s basal salts (MBS) medium (pH 7.5) supplemented with microcrystalline cellulose as a sole carbon source. The culture was incubated at 50°C, and was maintained by continuous sub-cultivation for enrichment of cellulase degrading microorganisms. The enrichment culture was then diluted and spread onto MBS plates supplemented with microcrystalline cellulose, and the plates were incubated at 50°C. Some of the colonies on the plates were purified by single-colony-isolation. The isolates were examined their activities of cellulase production.

A total of 38 isolates were purified by single-colony-isolation. Among these isolates, 18 strains showed cellulase activity and 9 strains showed relatively strong CMCase activity. The 16S rDNA sequence analysis indicated that these 27 isolates were classified into four groups in species level. The two of the four groups showed less than 95% similarity with any published 16S rDNA sequence, and were related to the sequences of Paenibacillus spp. This result indicated that isolates of these two groups are novel species of the genus Paenibacillus or novel genus. The optimal temperature and pH of strain TSTC2-10 were 50°C and 8.2, respectively. Further studies of physiological and chemotaxonomic characterization will be necessary for final decision of taxonomic status of these isolates. Also, biochemical and enzymatic characterization of cellulases from isolates of SCAs3 and TC22-2b are now in progress.

Keywords: cellulose, cellulase, thermophilic bacteria

Isolation and identification of chitinolytic bacteria from pruning tree compost

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Chitin, a linear homopolymer of N-acetyl-D-glucosamine (GluNac), is the second most abundant polysaccharide in the biomass. Biological degradation of chitin is accompanied by endo- and exo-enzymes known as chitinase (EC 3.2.1.14) and β-N-acetyl-D-glucosaminidase (EC 3.2.1.52). Chitinases are digestive enzymes that break down glycosidic bonds in chitin. Because chitin composes the cell walls of fungi and exoskeletal elements of some animals, chitinases are generally found in organisms that either need to reshape their own chitin or to dissolve and digest the chitin of fungi or animals. Enzymes participating in chitin degradation are produced not only by organisms containing chitin in their body but also by bacteria, upper plants and mammals where chitin is not present on a regular basis. Bacteria produced chitinases play a significant role in maintaining the matter cycle through making chitin usable biologically. Also, bacterial chitinases have a potential to use industrial chitin degradation. In our recent attempt to find chitinolytic bacteria, we successfully isolated moderately thermophilic bacteria degrading colloidal chitin.

Chitinolytic bacteria were isolated from compost of pruning tree from Tochigi Prefecture, Japan. The compost was industrially fermented about 3-4 years, and was 55°C, pH 7.8. The compost was suspended in saline, and the supernatant was inoculated into modified Brock’s basal salts (MBS) medium (pH 7.5) supplemented with chitin powder (2%, w/v), and incubated at 50°C for enrichment of chitin degrading bacteria. In these primary enrichment cultures, the growth of microorganisms was observed, among them cocci and rods of various lengths. The enrichment culture was then diluted and transferred onto MBS-broth plates for isolation of the single-colonies. After one or two days incubation, single colonies were visible on the plates, and were round and white with their diameter varying from 0.2 to 1.2 mm. A total of 77 bacterial isolates were purified by single-colony-isolation. To examine the chitinase activity, the isolates were carried out by spot inoculum of each colony on plates containing MBS medium with 0.5% colloidal chitin (w/v) as a sole carbon and energy source, and incubated at 50°C. The chitin degrading organism formed clear zones surrounded by colonies indicating chitinase activity, and 15 isolates were the most potent chitinolytic bacterial species.

Identification of the investigated chitinolytic strains, nucleotide sequences of 16S rDNA genes were determined, and those 15 isolates were classified into three groups in species level. Out of these, we chose strains TSTC2-10, TSTC2-19 and TSTC2-20 as representatives of the thermophilic isolates. Strain TSTC2-19 and TSTC2-20 could be affiliated with Geobacillus stearothermophilus and Pseudoxanthomonas taiwanensis, respectively. On the other hand, the 16S rDNA gene sequence of strain TSTC2-10 showed less than 93% similarity with any published sequences, and was related to the sequences of Paenibacillus spp. This result indicated that strain TSTC2-10 is novel species of the genus Paenibacillus or of novel genus. The optimal temperature and pH of strain TSTC2-10 were 50°C and 8.2, respectively. Enzymatic properties and quantitative measurement of the chitinase activities of these isolates are now in progress.

Keywords: chitin, N-acetyl-D-glucosamine, chitinolytic bacteria, chitinase, thermophilic
Isolation of Feather Degrading Bacillus spp. from Poultry Waste that Produce Keratinase in Iran

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Introduction: Feather constitutes over 90% protein, Feather-degrading bacteria produce keratinase. Keratinases from microorganisms have attracted industrial applications such as in the feed, fertilizer, detergent, leather and pharmaceutical industries. Aim of this study isolation of feather degrading Bacillus spp. from Iran.

Material and method: Bacillus strains isolated from poultry grown in basal media with feathers as its primary source of carbon, nitrogen, sulfur and energy, that incubate in pH 7.4 and 26°C for 7 days. Isolates identified as Bacillus spp. with physiological and biochemical tests and PCR for 16srRNA. Then keratinolytic active and optimal conditions for the enzyme production was done.

Result: seven strains of Bacillus were isolated from poultry waste in Iran. Among those isolates and identified as Bacillus pumilis, B. subtilis, B. firmus, B. larvae, B. popilliae, B. lentimorbus that degraded feathers effectively. Among these isolates, B. subtilis shown the maximum keratinolytic activity and optimal conditions for the enzyme production were 40°C and pH 5.8.

Discussion: Keratin-degrading microorganisms thrive under different ecological and environmental conditions and are known to have the capacity to solubilize keratinous substrates. Moreover, Bacillus strains are thermophile microorganisms and this property can be used in controlled process for efficient and fast degeneration of feathers.

Keywords: keratinase; bacillus; poultry waste

Kinetic and metabolic characterization of a mezcal-mash-isolated yeast growing in sugar cane bagasse hydrolysates for ethanol production.

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Current demand on energetics involves the search for environmentally sustainable alternative energy sources, in order to satisfy the needs of industry and population that cannot longer be affordable with fossil fuels. A strategy to reduce the consumption of crude oil and environmental pollution is the use of Bio-fuels. Ethanol can be obtained by the fermentation of sugars, products from starch and celullosic biomass. Celullosic resources (paper, wood, agricultural wastes) are very widespread, abundant and cheaper. In order to use celullosic compounds as carbon and energy source it must be hydrolyzed before fermentation. This process is carried out by an enzymatic complex called celloholocellulas (celulases and xylanases). Oligosaccharides, product of the enzymatic hydrolysis, are then used as carbon source for a wide range of fermentation processes. The aim of this work was to establish growth conditions of a native strain of yeast isolated from mezcal mash in a complex culture media with sugarcane bagasse hydrolysates. In order to identify metabolic end-products during fermentation, compare the ethanol production yield with other microorganisms reported in literature.

The yeast was activated in a complex medium containing (g·L⁻¹) 3 malt extract; 5 bactopeptone; 10 total sugars from sugarcane-bagasse-enzymatic hidrolysate. Sugars were obtained by hydrolysis of sugarcane bagasse with cellulases and xylanases of Cellulomonas flavigena mutant PR-22. To establish the optimal parameters of growth and ethanol production, four assays were conducted simultaneously in stirred tank bioreactors of 500 mL with an operation volume of 380 mL. The content of total soluble sugars in the sugarcane bagasse hydrolysate was determined by the technique of Dubois. All fermentations were carried out as batch cultures during 72 h. Samples of 1.5 mL were taken every 2 hours to monitor kinetics. The amount of total sugars, reducing sugars, inorganic nitrogen, ethanol, protein and biomass were quantified in each sample. Reducing sugars were measured by DNS technique; inorganic nitrogen was measured by Nessler technique; ethanol concentration was determined by Conway micro diffusion technique; proteins were quantified by Lowry method and biomass was estimated by dry weight.

It was observed that yeast could growth in both aerobic and anaerobic conditions; however the yield Yx/s for each condition was different (0.345 and 0.25 g·g⁻¹ respectively). Nevertheless biomass production was done, at the end of each culture, the ethanol concentration was 7.1 g·L⁻¹ for both conditions. Aerobic cultures increased the pH, while anaerobic cultures decreased this variable. Protein concentration in medium remained in the interval of 4 - 5 g·L⁻¹. These results gave us a general overview of the metabolism of the yeast. The amount of ethanol and the biomass produced in both aerobic and anaerobic conditions, places the yeast in the group of the facultative aerobic microorganisms, although ethanol production yield obtained was lower than Saccharomyces cerevisiae, Zymomonas mobilis or Escherichia coli recombinants. Complex composition of substrate should be highlighted since not many native microorganisms are able to ferment this kind of sugars (cellobiogasaccharides). This work is a base for further experiments to describe yeast fermentative mechanism and the optimization in ethanol production. Phylogenetic identification of yeast is a goal to reach in this research.

Keywords: yeast; saccharified; sugar cane bagasse; ethanol
Metabolically engineered *E. coli* gene expression: Efficient conditional gene silencing can be achieved using artificial convergent transcription protected from Rho-dependent termination


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The regulated redistribution of metabolic fluxes into branched pathways is extremely important for microbial biotechnology when biosynthesis of a desired product leads to growth retardation and decreased activity of the producing strain. Switching from the bacterial growth stage to the bioconversion of substrates into products of practical interest could be based on inducible expression of recombinant pathway genes or on conditional silencing of some key metabolic genes.

In the present study, we show that artificially arranged convergent transcription could be efficiently used for the conditional silencing of target genes. PykF is one of two pyruvate kinases in *Escherichia coli* K-12. ΔPykF was convergently integrated into the chromosome of the MG1655 strain, downstream of pykF and face-to-face with its native promoter. In the presence of the indicated cI*ts857, efficient pykF silencing was achieved when the 5′-terminus of the origin of antisense RNA (asRNA), consisting of the rrnG-AT sequence, converted elongation complexes of RNA polymerase to a form resistant to Rho-dependent transcription termination. pykF silencing was detected by the following features: (i) impaired growth of the strain when pykA was also disrupted and when using ribose as a non-PTS carbon source; (ii) reduced synthesis of full-sized pykF mRNA detected by reverse-transcription PCR; and (iii) a significant decrease of PykF activity. The advantages of anti-terminated convergent transcription were clearly manifested in strains where the rho_a-terminator was inserted specifically to interrupt asRNA synthesis. The target gene was most likely silenced by transcriptional interference due to collisions between converging RNA polymerases, although the role of cis-asRNA effects could not be excluded. While details of the mechanisms have yet to be determined, anti-terminated convergent transcription is a promising new technique for silencing other target genes.

**Keywords** Elongation complex collisions, gene silencing, *rrnG* anti-terminator, transcription interference

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Method of Lysine extraction from the culture fluid of producent

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The subject of this inquiry is method of Lysine extraction from the culture fluid of producent (*Brevibacterium sp. 90H*), which includes Lysine extraction from the culture fluid by the centrifugal extractors and its reextraction for further obtaining of crystalline lysine with concentration of 96-98 % of main substance.

Lysine extraction was carried out directly from the untreated culture fluid obtained from fermenters at centrifugal extractors, while reextraction was carried out by gaseous ammonia in the presence of small quantity of water. The culture fluid was acidified by sulfuric acid to the pH 1.8 and the extraction was carried out by dinonylnaphthalene solution in ammonia form at centrifugal extractors “EC-140” (Russia).

For the total lysine extraction culture fluid and extragent were moving in back flow to each other. 5 joined in series extractors were used to carry out the extraction. Culture fluid was introduced in the first extractor, and extragent – in the last one. As a result, maximal saturated lysine extract was obtained from the first extractor, and maximal “delysined” cultural fluid. For the reextraction, lysine containing extract was treated with the gaseous ammonia.

**Keywords**: lysine, extraction, culture fluid
Microalgae from The Salar de Atacama (Northern Chile), as a potential resource of fatty acids of industrial interest

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Microalgae are identified as an important resource of insaturated fatty acids, phospholipids, glycolipids and carotenoids, which are useful compounds for the alimentary and pharmaceutical industry.

The aim of this study was to obtain and identify fatty acids from microalgae strains isolated from altiplanic aquatic ecosystems at Salar de Atacama, northern Chile. From a total of seven isolated strains, Dunaliella salina, Chlorella sp. and Oscillatoria sp. were cultured in containers of 20 l under continuous aeration and lighting. The extraction of total lipids from pellet of each microalgae was carried out by the Bligh and Dyer method. Lipidic extracts were separated by Thin Layer and Gas Chromatography. From the total lipids, the analysis identified the 36,7%, 50,3% and 23,6% of them in D. salina, Chlorella sp. and Oscillatoria sp., respectively. With regard to the fatty acid composition in the total lipidic extraction, it stands out the 27,68% of stearic acid and 19,12% of oleic acid (C18:1 cis 11b) and 3,05% of stearic acid (C18:0) were obtained from Chlorella sp. and Oscillatoria sp., respectively. Also, palmitoleic acid (C16:1 cis 9) (28,53%) and linoleic acid (C18:2ω6) were detected in Oscillatoria sp., whose values are higher than those in the literature. We compare the fatty acids obtained in this work with data for microalgal strains of the same Genera reported in literature. The results obtained suggest that the fatty acids content and the composition of microalgae are related to the strain and their ecological niche, due to changes in response to environmental variables. The microalgal strains used in this work inhabit in extrem systems, with regard to temperature, light and salinity. On another side, fatty acids as C18:0 and C18:1 are recognized as carotenoids promoters, which are important pigments for the irradiance protection. More studies are necessary to clarify the relationship between the particular high fatty acid content in these microalgae and their role as promoters of metabolites necessities to life in extreme conditions.

This study was supported by a Grant ESO AUI 2006-2008.

Keywords: Microalgae; Gas Chromatography, Fatty acids

References:

Microbial Adaptation to Toxic Organic Solvents– Mechanisms and Biotechnological Applications

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Many organic solvents also known as potential environmental pollutants such as monoaromatic compounds (e.g. BTEX and phenols), n-alkanols and terpenoids are known to be extremely toxic not only to humans, animals and plants but also to microorganisms that are capable to degrade them. Therefore, environments contaminated with high concentrations of such compounds cannot be effectively bioremediated due to the inhibitory effects of the pollutants on the microbiota.

In addition, the toxicity of organic solvents plays a role in the biotechnological production of fine chemicals in whole cell biotransformations. One major problem of successful applications of biotransformations using living bacteria as biocatalysts is the high toxicity of potential substrates and products. Fine chemicals often show a hydrophobicity very close to those of biological membranes. For that reason such substances have toxic effects on the cell membranes and disturb their functioning, lead to growth inhibition or even cell death. These facts limit the economic application for biotechnological syntheses of a broader range of fine chemicals. One possible solution of this problem could be the application of fermentation systems with two phases. Hereby, an organic solvent is added as a second phase which functions as a kind of reservoir for potential substrates and/or products within a biotransformation process. However, applicable solvents are often toxic to potential biocatalysts themselves.

A solution for this problem is the application of so-called solvent-tolerant bacteria, often belonging to the genera Pseudomonas, Rhodococcus and Arthrobacter. These bacteria are able to adapt to supersaturated concentrations of many toxic solvents and environmental pollutants, respectively. In the last two decades, many attempts have been made to elucidate and characterise the cellular mechanisms of these bacteria enabling them to adapt to such hazardous conditions.

The main purpose of the lecture will be to introduce into the physiology and biochemistry of such solvent-tolerant bacteria and their specific adaptive mechanisms. On the other hand, potential applications of these special bacteria in the fields of bioremediation, environmental biotechnology and biocatalysis will be presented.

Keywords: solvent tolerant bacteria; adaptation; two-phase fermentations; fine chemicals; whole cell biotransformations

References:


Microbial Enzymes - An Alternative to Harsh Chemicals in Industry

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Currently enzymes have attracted the attention of the world due to their wide range of industrial applications in many fields including organic synthesis, clinical analysis, pharmaceuticals, detergents, food production and fermentation. Protease represents one of the largest groups of industrial enzymes and accounts for about 60% total worldwide sale of enzymes. Microorganisms are considered as a good source of enzymes due to their broad biochemical diversity and susceptibility to genetic manipulation. Using the growth condition of Streptococcus macedonicus, nine extracellular protease producing bacteria were isolated from different sites of East Calcutta Wetland (ECW), which acts as the world’s largest natural treatment plant for solid and soluble wastes located at the eastern edge of Calcutta. It is an excellent example of integrated resource recovery systems where city sewage is used for fisheries and agriculture for more than 100 years now. In ECW the bioremediation and biodegradation of complex compounds is mainly based on microbial activity, so it is recognized as a source of biotechnologically important bacteria. The early study on biodiversity screening of this area based on culture independent approach revealed the existence of wide variety of microbial population. Based on this background study nine extracellular protease producing bacteria were isolated from various sites of East Calcutta Wetland.

Most of the proteases from the bacterial isolates were found to tolerate a wide range of pH but all of them worked best at moderate range of pH. The thermo tolerant nature of the enzymes from the different strains varied between 4°C to 60°C. Extracellular protease from one of our novel bacterial isolate had shown the capability of enhancing the cleaning efficiency of detergent. It showed compatibility with different types of commercially available detergents and worked on different types of fabrics. Protease from another strain was working efficiently as dehairing enzyme at neutral pH and it is comparable to the conventional method of dehairing. Microbial proteases are used in leather industry for dehairing and bating of hides. The conventional method of leather making resulted in the huge amount of pollution due to chemicals like lime, sodium sulphide, salts, solvents comes from the leather processing. As the protease from our strain works at a neutral pH, it can reduce the use of environmentally hazardous chemicals used in dehairing process. Another novel strain was found to produce another industrially important enzyme amylase and three of the isolates were found to produce the enzyme lipase. Thus the isolated bacterial strains could be exploited for different industrial processes.

Keywords: Extracellular protease; Commercial application

Microbial production of lactate-based polyesters

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Bio-based plastic has been of interest because they can be an environmentally friendly alternative to petroleum-derived plastics. PLA is one of the most widespread bio-based plastic. Currently lactate-based polyesters were produced via microbial fermentation of lactate and chemical synthesis by ring-opening polymerization. Our group recently constructed a novel lactate-based polyesters producing system with one-step bioprocess (see figure below) using bacterial polyester biosynthetic system [1]. It has been known that many bacteria accumulate high-molecular-weight polysters, called polyhydroxyalkanoates (PHAs), as intracellular granules. A key enzyme of the PHA biosynthesis is PHA synthase, which polymerizes monomers into polymer. Typically, PHA synthases incorporate 3-hydroxyl and 4-hydroxyl acids into polymer. However, no enzyme had been reported to be capable of incorporating 2-hydroxyl acids, including lactate. Recently, we obtained a mutated enzyme, which incorporates lactyl unit into polyester chain, through the engineering of PHA synthase [2]. The discovery of the “lactate-polymerizing enzyme” was a breakthrough to construct the bioprocess producing lactate-based polyesters.

We constructed a microbial process producing lactate-based polyesters using Escherichia coli as a platform. The cells expressing the lactate-polymerizing enzyme and monomer supplying enzymes produced P[lactate-co-3-hydroxybutyrate (3HB)] copolymers comprised of 6—47 mol% lactate from glucose [3]. In addition, lactyl unit incorporated into polymer was all R-form (D-lactate) based on enantioselective HPLC analysis. The high optical purity should be an advantage against chemically synthesized polyesters. The new microbial process has a potential to be an environmentally friendly system for lactate-based polyester production.

Keywords: Biosynthesis; Enzymatic polymerization; PLA; polyhydroxyalkanoate synthase

References

Keywords: Biosynthesis; Enzymatic polymerization; PLA; polyhydroxyalkanoate synthase
Microbiological aspects of water retting in kenaf (Hibiscus cannabinus) processing

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During the last recent years a renewed interest has occurred in the production of natural plant fibers. This production can be achieved naturally by the so-called “retting” process which relies on the exploitation of specific enzymatic activities that microbial communities associated to the plant biomass are able to exert. Traditionally, two retting methods - namely “dew retting” and “water retting” - have been adopted. Through these treatments, depolymerization of the pectic cement enrusting fibers is carried out by bacterial (mostly in “water retting”) or fungal (especially in “dew retting”) pectinolytic enzymes which bacteria colonizing the epigous portions of the plant species or soil occurring microorganisms release outside the cells. Nevertheless, the difficulty so far experienced in the control of the retting process has represented the major limitation to an efficient production of high quality fibers. Therefore, this aspect must be taken into account as the key issue for any future expansion of industrial fiber crops. Since microorganisms are the main retting agents, right their trophic, eco-physiological and biochemical properties end to affect the course of the pectinolytic process and, as a consequence, the final quality of the fibers obtained. For this reason, to better understand the microbial retting reactions results of crucial importance. Nowadays, studies on retting of different fiber crops (particularly hemp and flax) lead to assume a dynamic succession of different pectinolytic microbial species during the process. Actually, Bacillus spp. are dominant in the starting phase of the process, while spor-forming anaerobic Clostridium spp. take place with the progressive depletion of oxygen in water tanks. Although Clostridia are considered to be the major group of bacteria responsible for the traditional water retting, they can however negatively affect the quality of the end-product in long lasting retting cycles, due to their capability to degrade even cellulose that is the main fiber component.

The aim of the present study was either to assess the main factors driving the retting process or to decipher the microbial community responsible for the depickination of kenaf (Hibiscus cannabinus) fibers in a pilot bioreactor. Kenaf, is a plant species belonging to the botanical family of Malvacae and represents one of the allied fibers of jute. The pilot bioreactor consisted in a 1 m³ tank featured with an outer water jacket for the temperature control, a recirculating pump for the continuous mixing of the retting liquor, and an air insufflation system for the delivery of oxygen into the fermenting liquor. A series of sensors were set in order to check temperature, pH, redox potential, and oxygen concentration in the bioreactor. The reactor was connected to a computer for the continuous monitoring and the control of the process parameters as well as for the storage of data. The diachronic evolution of the retting microflora was evaluated by two distinct but complementary approaches: i) a culture dependent approach based on the isolation - on selective growth media - of bacteria colonizing the fibers or acclimated in the retting liquor inside the reactor; ii) a culture independent approach consisting in a metagenomic analysis of the retting liquor by PCR-DGGE protocol performed on universal 16S rDNA with Bacillus-specific primers. The culture dependent approach revealed that the facultative anaerobic microflora – including both sporogenous and non-spor-forming species - is mainly responsible for plant tissue maceration. Spore-forming bacteria were prevalently Bacillus spp. that evidenced a good pectinolytic activity through esoppectinase production. On the other hand, non-spor-forming bacteria, chiefly represented by Proteobacteria, evidenced low esoppectinase production along with significant pectin fermentation ability, as well as good tolerance to phenolic toxic compounds generated during plant tissue maceration. Interestingly, culture dependent PCR-DGGE analysis confirmed the results gained through culture dependent protocols. Molecular analyses allowed attributing the isolates to different steps of the retting process. They also revealed the presence of Clostridium spp. in the bioreactor. As a matter of fact, running kenaf retting in the aerated bioreactor pointed out a continuous presence of Bacillus spp. during the whole process and allowed to understand how to control the presence of Clostridium. Moreover, a new trait of the retting process was revealed: the occurrence of Proteobacteria. This investigation represents an innovative approach to the study of wet retting microflora in toto whose components were taxonomically identified and characterized for their role within the process in a controlled pilot bioreactor.

Microbiological characterization and disposal issues of table olive wastewaters

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The preparation of edible olives is an important process of the olive agro-industry. In Greece, the processing of Greek-style naturally black olives includes harvesting, washing and placement in brine containing 8-10% v/v salt and often vinegar. Despite the fact that research on table olive quality and fermentation has been extensively performed in the last decades, no information on problems due to the disposal of brine after packaging has been reported. In the present work, the wastewater of table black olives (variety Kalamon) was evaluated in terms of its phytotoxicity as well as its physico-chemical and microbiological properties. Physicochemical analyses and phytotoxicity were performed according to standard methods, while microbiological investigation of the disposed brine was performed by constructing a clone library. This saline wastewater had a low pH of 4.2, high COD (33.4 g l⁻¹) and phenolic content (5.3 g l⁻¹). No germination was occurred in 25 and 50% v/v olive brine, while phytotoxicity was greater than 99% in 10% v/v brine solution. The majority of environmental clones obtained were placed within the α- and β-Proteobacteria, while no lactic acid bacteria were present. Moreover, 16S rDNA gene sequence analysis revealed no threat from any potential pathogen.

Keywords: saline wastewater, brine, table olives
Mixture design of agricultural waste substrates for laccase production from white rot fungal

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The objectives of this study were to find out the best substrates for laccase production in solid state fermentation and determined the best model of mixture design of three selected substrates. The method used ten agricultural waste as substrates for laccase production in solid state fermentation from white rot fungal, WR 710-1 collected from Hang Dong district, Chiang Mai province, Thailand. The highest laccase activity (0.688±0.117 U/g substrate) from WR710-1 when grew on orange peel. Laccase activities 0.171±0.028 U/g substrate and 0.160±0.044 U/g substrate were found when WR710-1 grew on corn cob and coffee husk, respectively. Three selected substrates, orange peel, corn cob and coffee husk were mixed in mixture design to determine the model of the mixtures. The best model was \( Y = 1.129x_1 + 0.164x_2 + 0.356x_3 + 3.741x_1x_3 \) with Coefficient of Determination \( (R^2) \) as 0.884. Moreover the maximum activities of laccase (1.974±0.120 U/g substrate) was increased 2.85-fold when fermented in mixed substrates using this experimental model.

Keywords laccase; mixture design; white rot fungi; agricultural waste

Molecular Characterization of Escherichia coli (E. coli) mercuric reductase (merA) gene.

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Studies related to geographical distribution of E. coli carrying mercuric reductase (merA) gene were carried out on the Indian subcontinent. Out of 30 E. coli isolates, collected from five geographically distinct regions of India, 8 were found to be resistant to the inorganic form (HgCl₂) and only 2 to resistant to both the inorganic and organic forms of mercury. All the 8 strains revealed the presence of a plasmid of 24kb, and transformation of the isolated strains into the mercury-sensitive competent cells of E. coli DH5α rendered the transformants resistant to the same concentration of mercury as wild type-strains.

On PCR amplification of mercuric reductase (merA) gene, the expected length of PCR products of merA genes corresponding to 1695bp was obtained from all the 8 strains when electrophoresed on 1% agarose gel. The PCR products of 2 highly resistant strains were cloned in pGMET-Easy Vector. An analysis of the nucleotide sequence of the merA genes of the E. coli isolated from 2 highly resistant strains showed remarkable degree of homology, except for a few nucleotide base changes. At restriction level, these merA genes showed divergence from each other. These variations were exhibited in terms of restriction sites for different restriction enzymes.
Multigenic family coding for endo-1,4-beta-xylanases in *Penicillium canescens*

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There are numerous microorganisms efficiently degrading xylan, a major component of lignocellulose. In particular, filamentous fungi have demonstrated a great capability to secrete a wide range of xylanases. A great number of *Penicillia* are active producers of xylanolytic enzymes and xylanases from these species have many commercial uses in biotechnological applications. Several of them produce multiple isoforms of xylanases. Commonly xylanases belongs to glycoside hydrolase families 10 and 11. The filamentous fungus *Penicillium canescens* secretes high level of xylanase, which gets a good potential for biobleaching of cellulose pulp. The complete gene Xyla coding for major family 10 endo-1,4-beta-xylanase Xyla (M, 31 kDa) of this organism was cloned and described earlier.

Four additional *P. canescens* genes, *XYLB*, *XYLC*, *XYLD*, and *XYLE*, encoding the XyIb, XyIc, XyId, and XyIe endo-1,4-beta-xylanases, respectively, have been cloned and sequenced. *XYLB* encodes a presumed signal peptide of 32 amino acids (aa) and a mature protein of 178 aa. *XYLC* – 31 aa signal protein and mature protein of 212 aa. *XYLD* - 27 aa signal protein and mature protein of 190 aa, *XYLE* - 35 aa signal protein and mature protein of 325 aa. Sequence alignment and the constructed neighbor-joining tree showed that the *P. canescens* enzymes XyIb, XyIc and XyId belongs to glycoside hydrolase family 11, XyIe belongs to glycoside hydrolase family 10. Coding regions of these genes are interrupted by one intron. All new endoxylanase proteins are closely related to several other xylanases from *Penicillia* and Aspergilli.

Several xylanase producing *Penicillium* species contain consensus sequences for CreA and XlnR binding in the promoters of the xylanase encoding genes. The promoters of cloned genes have been sequenced partially and analyzed. The promoter of *XYLB* gene has a modular structure, with seven putative XlnR binding sites in tandem (XlnR module), and upstream from them, four putative CreA binding sites (CreA module).

All genes have been expressed in a laboratory *P. canescens* strain under control of a strong native promoter, resulting in the construction of corresponding xylanolytic strains. Secreted endoxylanases demonstrate following properties: XyIb - an apparent M, 19 kDa and an isoelectric point below 6.07, XyIc - M, 22 kDa and an isoelectric point below 5.3, XyId - M, 21 kDa and an isoelectric point below 5.3, XyIe - M, 36 kDa and an isoelectric point below 5.2. Xylanase activities of these enzymes were maximal at pH 5.0-5.5 and 50-55°C, excepting XyId with temperature optimum 70°C.

Keywords xylanases; *Penicillium canescens*; glycoside hydrolase families; promoters; binding sites

Old Yellow Enzymes: Powerful biocatalysts for the asymmetric hydrogenation of C=C bond

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Old Yellow Enzymes (OYEs, enoate reductases) are now powerful and excellent biocatalysts for the asymmetric hydrogenation of the C=C bonds of α,β-unsaturated compounds. We found two novel OYE families (CYE and TYE) catalyzing the asymmetric hydrogenation of C=C bond with different substrate recognition mechanism, and these OYEs were applied to a bioreduction system for the production of double chiral compound.

I. OYE from *Candida macedoniensis* (CYE)

Microorganisms were screened for reducing ketoisophorone (KIP), and *C. macedoniensis* was found to produce (6R)-levodione through the asymmetric hydrogenation of the C=C bond of KIP [M. Kataoka et al., *Biosci. Biotechnol. Biochem.*, 2003, 66, 2651]. The enzyme involved in this reaction was identified as OYE family protein. The asymmetric reduction of KIP to (6R)-levodione was successfully done with *E. coli* transformant cells, in which both CYE and glucose dehydrogenase (GDH) genes were coexpressed. The (6R)-levodione formed amounted to 96.6 mg/ml (627 mM), with molar yield of 95.4% [M. Kataoka et al., *J. Biotechnol.*, 2004, 114, 1]. The resultant (6R)-levodione was converted to (4R,6R)-actinol for the commercial production of zeaxanthin and xanthoxin.

The simultaneous sequential conversion of KIP to (4R,6R)-actinol via (6R)-levodione with both CYE and LVR was performed. However, the main product of the reaction was (4S)-phorenol, suggesting that CYE catalyzes the asymmetric hydrogenation of only KIP, while LVR catalyzes the reduction of both (6R)-levodione and KIP (Fig. 1). Thus, (4R,6R)-actinol production with CYE and LVR must be performed through two separate reactions.

II. OYE from *Torulopsis* sp. (TYE)

Microorganisms were screened again as to the reduction of (4S)-phorenol, and *Torulopsis* sp. was found to produce (4R,6R)-actinol through the asymmetric hydrogenation of (4S)-phorenol. The enzyme involved in this reaction was also identified as OYE family protein, but TYE catalyzes the hydrogenation of both KIP and (4S)-phorenol (Fig. 1). Using cells of a single *E. coli* transformant coexpressing the TYE, LVR and GDH genes as the catalyst, KIP was almost stoichiometrically and stereospecifically converted to (4R,6R)-actinol through simultaneous sequential conversion.

Keywords Old yellow enzyme; bioreduction; asymmetric hydrogenation
Optimization of a fermentation process for butanol production using Particle Swarm Optimization

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Particle swarm optimization (PSO) was evaluated in relation to their capability to optimize an alternative fermentation process for the production of biobutanol. The process consists of three interconnected units, as follows: fermentor, cell retention system (tangential microfiltration) and vacuum flash vessel (responsible for the continuous recovery of butanol from the broth) (Figure 1). The dynamic behaviour of the process is described by a non-linear mathematical model with kinetic parameters determined experimentally, whose complexity make the solution of the optimization problem difficult through conventional deterministic algorithms, thus justifying the use of a heuristic method. Different PSO algorithms (Original, Trelea 1, Trelea 2, Shi-type, Clerc-type) were employed to determine the process inputs that maximizes the productivity of butanol constrained by a desired substrate conversion. According to the analyses of these algorithms, the performance of all PSO types was similar. With 10 iterations, PSO achieved a maximum value of butanol productivity of 9.21 g/l.h (Figure 2). Thus PSO showed to be suitable to optimize the fermentation process for butanol production. The use of PSO in biochemical processes, particularly in the butanol fermentation, is a novel approach and sums up the efforts of recent researches in turning the biobutanol industry commercially viable.

Optimization of a protoplast transformation method for *Bacillus Subtilis*, *Bacillus megaterium*, and *Bacillus Cereus* by a plasmid pHIS1525.SPlipA

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During the past years of gene cloning studies, *Escherichia coli* has always been a foremost host cell for exogenous genes expressions owing to its high level of protein production and excretion. However, problems relating to low level of extracellular production of some proteins specially the accumulation of cloned proteases within the cells have moved the attentions from *E.coli* to bacilli bacteria such as *B. megaterium*, *B.subtilis*, and *B.cereus* due to their sporulation capability of many different enzymes. *Bacillus megaterium* is widely used for high-level expression of *Bacillus subtilis* is widely used for high-level expression of *Bacillus megaterium* is widely used for high-level expression of heterologous proteins with little or no degradation. *Bacillus subtilis* is a naturally competent host cell for uptake of extrogenous DNA, resulting in attractive industrial applications. *Bacillus cereus* has sporulation capability which makes it suitable for several industrial uses.

A conventional approach for transferring DNA into protoplasts or intact cells of bacilli bacteria is chemical transformation, using chemicals through chilling and then shock-heating of the suspension of cells to induce reversible permeabilization of the cell membrane to make it possible for the external DNA to enter into the cells. In most cloning experiments, the transformation with plasmid DNA is performed using Polyethylene glycol (PEG)-induced competence cells.

In this study, a PEG-induced protoplast transformation protocol was developed for three different bacillus strains of *Bacillus megaterium ATCC®14945, Bacillus Subtilis ATCC®6051, and Bacillus Cereus ATCC®14579*. In all cases a plasmid pHIS1525.SPlipA, well working vector in *B.megaterium*, was applied. Protoplasts were formed in RHAF medium after treating the cells with lysozyme. Two factors, the incubation time and the lysozyme concentration have been found to play the most important role in effective protoplast formation. These two factors were further optimized in this study to elaborate a chemical transformation procedure which can possibly work for other bacillus strains as well. The optical density (A420) and the number of colony-forming units (CFUs) were determined to find the optimal conditions for each strain. The results indicate that PEG-induced protoplast transformation is a sufficient technique when using a plasmid pHIS1525.SPlipA in *Bacillus* genus.

**Keywords**: *Bacillus megaterium, Bacillus Subtilis, Bacillus Cereus, PEG-induced protoplast transformation*

![Fig. 1. General scheme of the continuous flash fermentation process](image1.png)

![Fig. 2. Evolution of the butanol productivity for each iteration](image2.png)

**Keywords**: flash fermentation, biobutanol, optimization, particle swarm optimization
Optimization of Dilute Acid and Alkali Pretreatment of Sweet Sorghum Bagasse for Microbial Saccharification Using Response Surface Methodology

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New transportation fuels are desperately needed to reduce our heavy dependence on fossil fuels and to slowdown global climate change. Lignocellulosic biomass is the only low cost, alternative energy source available for sustainable production of large quantities of liquid transportation fuels, through saccharification and then fermentation of the released reducing sugars &/or pretreated biomass. However, the digestibility of lignocellulosic biomass is low owing to its structural features such as lignin content, cellulose crystallinity, hemicellulose acetylation and inaccessible surface area, thus limiting the saccharification of cellulose and making pretreatment steps essential. Further, this is the most expensive lignocellulose processing step. Therefore, a proper pretreatment method is needed to overcome these problems and to achieve higher yields. Among various pretreatment methods investigated (biological, chemical, physical and thermal), at present only those employing chemicals, particularly dilute acid or alkali, offer higher yields and low costs vital to economic success.

Therefore, in present study a response surface methodology (RSM) was used separately for dilute (sulfuric) acid and alkali (sodium hydroxide) pretreatments of sweet sorghum bagasse, a low cost lignocellulose rich biomass to standardize the pretreatment conditions. The standardization was based on central composite rotatable design (CCRD) involving 4 variables acid/ alkali concentration, ratio of solid substrate and liquid solution (w/v), pretreatment temperature and pretreatment time at 5 levels each.

Results show that temperature had maximum effect on release of reducing sugars during acid pretreatment, followed by solid to liquid ratio and acid concentrations; however, during alkali pretreatment maximum effect was shown by alkali concentration. Acid pretreatment released 56.18 % (w/w) reducing sugars, compared to 1.32 % by alkali pretreatment. In conclusion, dilute acid pretreatment of sweet sorghum bagasse is more promising method than dilute alkali pretreatment for further microbial saccharification process, on the basis of released reducing sugars. Optimized conditions for acid pretreatment are: acid concentration- 1.4%, solid to liquid ratio- 1:10.9, temperature- 112 °C and time- 34.4 minutes.

Keywords: pretreatment; lignocellulose; central composite rotatable design (CCRD); response surface methodology (RSM)

Optimization of the Continuous Clavulanic Acid Adsorption Process

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This paper proposes an optimization of the variables involved in the continuous adsorption process of clavulanic acid (CA) on an ion exchange resin, using a complete factorial design. Six independent variables are involved: initial concentration of CA, residence time of adsorption tank, residence time of desorption tank, solid residence time of adsorption tank, and the liquid ratios in the two tanks. The factorial design was made utilizing 65 simulations of the continuous adsorption process obtained by a previously proposed model. The dependent responses, process yield (Y), purification factor (PF) and concentration factor (CF) were successfully optimized using the response surface methodology. A continuous adsorption run was done to validate the factors that maximized the process yield. A comparison of the Y, FC and FP values obtained in the simulated and experimental runs indicated a difference of less than 11%. The response surface methodology proved suitable to optimize the factors involved in the continuous adsorption process of clavulanic acid on the ion-exchange resin.

Keywords: clavulanic acid; continuous adsorption process, response surface methodology.
PCR clone of novel L-asparaginase II gene from *Escherichia coli* (YG 001)

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The interest in L-asparaginase arose of its anticancer activity. Unlike normal cells, malignant cells can slowly synthesise L-asparagine and are dependent on an exogenous supply. In contrast, normal cells are protected from asparagines starvation due to their ability to produce this amino acid. The anticanceractivity results from depletion of circulating pools of L-asparagine by L-asparaginase. In the present work, the L-asparaginase II (*ansB*) from *E. coli* YG 001 was cloned and expressed in *E. coli*.

The *E. coli* YG 001 was isolated during a screening program from urine and stool samples arrived to medical laboratory of the Namazi Hospital, Shiraz Iran. Total genomic DNA were isolated and used for PCR amplification of the *ansB* gene. Sequences were amplified using the specific primers, which amplify a ~1000-bp of the structural *ansB* gene. The resulting PCR amplicon was ligated in the *E. coli* expression vector. *E. coli* BL21 (DE3) cells were transformed with the recombinant plasmid to get the expression strain. The asparaginase activity was measured by Nessler’s reaction. The DNA and amino acid sequence of the L-asparaginase II of the *E. coli* YG 001 was recorded in the NCBI under the accession number GQ901077. The DNA and amino acid sequence alignments resulting from the BLAST search of *ansB* showed 95-99% sequence identity with the other strains of *E. coli*, whereas significantly lower identity was observed with other bacterial L-asparaginases II (75-80%). The *E. coli* transformants showed high asparaginase activity. The commercial availability of L-asparaginase II has revolutionized the molecular therapy of leukaemia. Thus, the characterization of new recombinant L-asparaginase II and the development of rapid, simple and effective production methods are not only of academic interest but also of practical importance.

**Keywords:** Gene cloning, L-Asparaginase, *Escherichia coli*

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PHA production by mixed culture from a by-product of paper industry

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Polyhydroxyalkanoates (PHA) are biodegradable biopolymers, which have been recognized as good candidates for synthetic polymers partial replacement. The greatest drawback for their use, as petroleum-based polymers substitutes, is the production cost, which can be four times higher than the chemical synthesis. PHA production by activated sludge is being investigated as a possible technology to decrease production costs, since no sterilization is required and bacteria can adapt quite well to the complex substrate present in low cost feedstocks. The most promising process using mixed microbial cultures (MMC) is based on the alternation of short periods of excess carbon (feast) with long periods of starvation (famine). One critical factor on the development of a competitive process for PHA production with MMC is the selection of organisms with high storage capacity.

Hardwood spent sulphite liquor (HSSL) is a by-product of paper industry that due to its high content in carbohydrates, such as xylose, can be converted by yeasts and bacteria to ethanol. One of the most efficient ethanol producers is the yeast *Pichia stipitis*. However the presence of acetic acid in HSSL is an obstacle to the conversion of xylose into bioethanol, since it inhibits the metabolism of *P. stipitis*. Researchers are looking for processes that remove acetic acid from HSSL and, preferentially, allow for an economic income. MMC can utilize volatile fatty acids (VFAs) like acetic acid for PHA production and were never described to be able to convert carbohydrates under aerobic dynamic feeding conditions (ADF). A sequential process was planned in order to, first the acetic acid is removed from HSSL and PHA are produced and then the effluent of this step, rich in xylose, is supplied to a *P. stipitis* system. The main objectives of this work were the selection of a culture presenting a stable PHA-storing capacity from acetic acid and to infer the possibility of using the effluent of this system for ethanol production.

The culture was selected in a sequenced batch reactor (SBR) under ADF conditions. After 36 days of operation the selected culture was able to produce 8% of PHA per cell dry weight. The microorganisms were able to uptake the acetic acid and convert it as poly-3-hydroxybutyrate. However a decrease in xylose in HSSL and simultaneous ethanol production was also observed. Under the microscope was possible to identify not only the bacteria involved in PHA storage (using Nile blue staining) but also the presence of a microbial contaminant with similar morphology to *P. stipitis*. The detection of xylose consumption and ethanol production and the fact that the ethanol system was operated in the same room could confirm that the contaminant was *P. stipitis*. Unfortunately due to type of operation of the SBR, it was not possible to quantify the maximum amount of ethanol produced in the SBR, since part of it was stripped by the air stream. The MMC was so efficient in removing the acetic acid from the HSSL that allowed for the survival of *P. stipitis* and bioethanol production. Consequently the hypothesis of utilization of a dual process for PHA and bioethanol production from HSSL was almost confirmed. The sequential process is under development.

The PHA storing bacteria community analysis in the system, selected under the applied operational conditions, was performed by Fluorescence In Situ Hybridization (FISH). If in the beginning of the system the typical genera found in ADF systems were scarcely present and only a part of bacteria was involved in PHA storage, around day 36 the population composition changed. It was possible to confirm the presence of bacteria belonging to *Azotobacter, Thauera* and *Zoogloea* genera.

**Keywords:** PHA; Mixed microbial cultures; HSSL, acetic acid
Physiologic diversity in Debaryomyces hansenii

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Debaryomyces hansenii is commonly recognized as a halotolerant, food grade (GRAS/QPS) yeast. It is used in the food industry (dairy and meat products) and may have many other potential biotechnological applications. It is also a very effective natural pentose assimilating yeast, and its use in the upgrade of the hemicellulosic sugar stream in a biorefinery framework holds a great promise. It can be used either as the microbial catalyst for the production of polyols such as xylitol and arabitol, as well as for the production of single cell protein or yeast extract, a key complex supplement for the economical viability of many envisage microbial based biorefinery processes, such as four Carbon 1,4-di acids (succinic, fumaric, and malic), 3-hydroxypropionic, glutamic, lactic, and acetic acids.

Nevertheless, it is commonly recognized that this taxa is a very diverse species. Actually, two varieties have been described, var. hansenii and var. fabryi, distinguishable by genetic methods, maximum growth temperature (higher for var. fabryi), and the presence (var. fabryi) or not (var. hansenii) of the glucose-6-phosphate dehydrogenase enzyme, differences that together with more recent genetic data lead to the proposal of re-establishing the species D. hansenii and D. fabryi, with apparently no other phenotypic criterion that distinguish them. This diversity can be an interesting source of potential biotechnological relevant traits that should be explored.

In this work, a total of twenty-eight D. hansenii strains obtained from several culture collections and laboratory isolates were characterized towards its metabolism of hemicellulosic-derived sugars. The strains were first grown in shake-flask with chemically define media (YNB w/o aminocids) using D-xyllose as sole carbon and energy source, at 28°C in semi-aerobic conditions, previously found to be relevant for the xylitol production process. Cultures were followed regularly for 48 h to evaluate sugar consumption, as well as biomass and metabolite production in order to enable the calculation of the comprehensive kinetic and stoichiometric parameters. Biomass was also recovered for a subsequent characterization of the activity of selected enzymes.

Fermentation profiles varied greatly, but two major groups could be found (A and B). These groups differ mainly on the xylitol assimilation, and on the biomass specific growth rate, yield and productivity, far higher in group A, than in B. These groups were further validated by cluster analysis that grouped all previously classified D. hansenii var fabryi and var. hansenii in different groups, A and B, respectively, which support a correlation between variety and pentose metabolism, probably linked to the differences on the glucose-6-phosphate dehydrogenase enzyme.

A second set of fermentation assays were carried out, now using complex nutrients (peptone, and yeast and malt extract) supplemented medium. It was observed a relief of the constraints of group B strains to xylitol metabolism improving fermentation performance, and thus presenting profiles comparable to non-supplemented group A strains. This points to an additional difference related to nutrient requirements within both groups whose specific nature is currently under study.

Overall kinetic and stoichiometric parameters for xylitol production process by these strains are compared and their implications for the development of microbial process for the valorization of the hemicellulosic fraction at industrial scale will be discussed.

**Keywords** Debaryomyces hansenii var. hansenii, Debaryomyces hansenii var. fabryi, xylitol, lignocellulosic materials, hemicellulosic, biorefinery.

Authors are grateful to Fundação para a Ciência e a Tecnologia (FCT) for the financial support of this work (project XilitolOut PTDC/AGR-AGB/71792/2006). Dr. Amanda Pires Pinto (INETI-DTIA, Lisboa, Portugal), Prof. Conceição Loureiro-Dias (ISA-UTL, Lisboa, Portugal), ARS Culture Collection (National Centre for Agricultural Utilization Research, Peoria, IL, USA) and PVCC (Portuguese Yeast Culture Collection, Almada, Portugal) are gratefully acknowledged for supplying yeast strains.

Physiological characterization of mannitol overproducing strains in carob based medium

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Microbial production of mannitol has become more attractive as some microorganisms can specifically produce mannitol from glucose/fructose mixtures without making sorbitol, a typical by-product of the non-specific reduction that occurs in the industrial chemical based method when using these type of substrates. Heterofermentative lactic acid bacteria (LAB) are among the best microorganisms with the ability to convert fructose into mannitol with high efficiency. Metabolically, under the adequate oxygen availability conditions, glucose can be used as an energy and carbon source and fructose as an electron acceptor, as it can be reduced to mannitol by means of a specific mannitol dehydrogenase (E.C. 1.1.1.67). Depending on the microorganism, up to two moles of mannitol can be produced from 1 mol of glucose (2 mol of fructose), if sugar assimilation is simultaneous. This will also lead to the formation of lactic and acetic acids and/or ethanol as other metabolic products.

In a previous work [1] thirty bacterial strains from Lactobacillus, Leuconostoc and Weissella genus were screened for mannitol production in carob-based syrups. From these, a group of eight strains which included Leuconostoc citreum ATCC 4970, Lc. mesenteroides subsp. cremoris ATCC19254, Lc. mesenteroides subsp. dextranicum ATCC 19255, Lc. fructosum NRRL B-23447, Lc. fructosum NRRL B-2041, Lc. lactis ATCC 19256, Lactobacillus intermedius NRRL 3692 and L. reuteri DSM 20016 were identified as potential mannitol overproducers.

In this work, a detailed kinetic and physiological characterization of these strains is performed using a carob based culture media, supplemented with the same nutrients as the classical MRS medium in order to evaluate their different metabolic capabilities. Cultures were thoroughly followed for 30 h to evaluate sugars consumption, as well as biomass and metabolite production. Fructose and glucose were always simultaneously consumed but fructose assimilation rate was always higher. The results obtained enable to divide the studied strains mainly into two groups: one for which glucose assimilation rates were always below 0.78 g/l.h (strains ATCC 4970, ATCC 19256 and ATCC 19254) and the other for which it ranges between 1.41-1.89 g/l.h (strains NRRL B-3692, NRRL B-2041, NRRL B-23447 and DSM 20016). These groups also exhibited different mannitol production rates and yields, being higher for the faster glucose assimilating strains. All strains produced mannitol at high yields (> 0.70 g mannitol/g fructose) and volumetric productivities (> 1.31 g/l.h). The best performance was obtained for Lc. fructosum NRRL B-2041 with a maximum volumetric productivity of 1.82 g/l.h and a stoichiometric conversion of fructose to mannitol. Fast glucose assimilating strains such as Lc. fructosum always have a mannitol to other products ratio above 1. This, together with the exhibited robustness and reproducibility led us to select Lc. fructosum NRRL B-2041 for further mannitol process optimization in carob-based media.

**Keywords** carob, lactic acid bacteria, Lactobacillus, Leuconostoc, mannitol production

[1] Moniz, P. et al. Screening and characterization of lactic acid bacteria for the production of mannitol in carob based syrups. This meeting.

This work was supported by Adl, Project ValorAlfa (70/00326). ARS Culture Collection (National Centre for Agricultural Utilization Research, Peoria, IL, USA) and Dr. Isabel Fernandes (INETI-DTIA, Lisboa, Portugal) are gratefully acknowledged for supplying bacterial strains.
Production of xanthan gum by \textit{Xanthomonas campestris} and Optimization its production process

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\textbf{Intruduction:} Xanthan gum is a heteropolysaccharide produced by the plant-pathogenic bacterium \textit{Xanthomonas campestris pv. campestris}. Xanthan gum is used in a broad range of industries, such as in foods, toiletries, oil recovery as emulsifier and stabilizers. Aim of this study is production of xanthan gum and optimization its production process.

\textbf{Materials and methods:} \textit{Xanthomonas spp}. were isolated from infected Cabbage leaves. For isolation, microorganisms were grown on nutrient starch cycloheximide agar. Adding nitrofuratoin, vancomycin and cephalixin reduced the development of saprophytic bacteria. For xanthan production, microorganisms were grown on YDC agar slant and then inoculated in broth medium that consists of (g/l) glucose 25, KH\(_2\)PO\(_4\) 15, K\(_2\)HPO\(_4\) 10, MgSO\(_4\).7H\(_2\)O 0.2, citrate 3, (NH\(_4\))\(_2\)SO\(_4\) 2.6 and FeCl\(_3\).6H\(_2\)O 0.00012 and incubated for 48 h at 25-35\(^\circ\)C.

After fermentation xanthan gum was recovered and its concentration was determined. Result: 12 Strains of \textit{X. campestris} were isolated. Among these isolates \textit{X. campestris} is having the ability for xanthan production at higher concentration, which are having commercial significance.

\textbf{Keywords:} Xanthan gum, Xanthomonas campestris, fermentation media.
Purification, immobilization and application of tannase for beverage clarification

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Tannase was produced under solid state fermentation using Jamun (Syzygium cumini) leaves by Aspergillus ruber. Tap water was used as moistening agent with 1:2 ratio and pH 5.5 and incubated at 30°C. No external carbon and nitrogen source was used for tannase production. Tannase was harvested after 96 hours and purified using ammonium sulfate fractionation and ion-exchange chromatography. The purified tannase was immobilized using gel entrapment method. Immobilized tannase showed more pH stability, more thermal stability and can be recycled. The purified enzyme was used to clarify the haze beer, wine, tea extract and orange juice. Tannase effectively reduces the haze in all the beverages within 4 hours of incubation at room temperature (30°C). The immobilized enzyme is stable even after one year at 4°C and can be further exploited for effective haze removal in the beverage industries.

Relations between methanol metabolism pathway enzymes and \(\beta\)-galactosidase activities during a fed-batch fermentation of \textit{Pichia pastoris}

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\textit{Pichia pastoris} is a widely used yeast species for the production of recombinant proteins. Because the volumetric productivity of a given recombinant production process is generally proportional to the density of biomass generated, the ability to easily grow this organism at ultra-high cell densities using methanol as the sole carbon source is a major advantage. But, probably the most important characteristic of \textit{P. pastoris} as host microorganism is the existence of a strong and tightly regulated promoter from the alcohol oxidase 1 gene, \textit{AOX1}. Alcohol oxidase (AOX) is the first enzyme of the methanol assimilation pathway which catalyses the oxidation of methanol to formaldehyde. Formate dehydrogenase (FDH) is an enzyme involved in dissimulation of excess formaldehyde produced by the AOX, which also has activity the incapacity for formaldehyde assimilation.

In this study, a recombinant \textit{P. pastoris} strain (GS115/His\textsuperscript{+} Mut\textsuperscript{+}), intracellularly producing the enzyme \(\beta\)-galactosidase, was used to investigate the relations between AOX and FDH activities and recombinant protein production during fed-batch fermentations. The \textit{P. pastoris} fed-batch fermentation process on methanol for attaining high cell-density consists of three distinctive phases; the glycerol batch phase for initial cell growth, the glycerol fed-batch phase for \textit{AOX1} derepression and increased cell density, and the induction phase for expression of recombinant proteins. The feeding strategies used in the glycerol and methanol fed-batch phases were based on the assumption of a constant specific growth rate of 0.18 and 0.03, respectively. pH was controlled at 5.0 and the temperature at 28 °C. \(\beta\)-galactosidase activity was assayed by a spectrophotometric stop rate method according to Miller (1972) using ONPG as a substrate. AOX activity was assayed by a continuous spectrophotometric rate method according to Keesey (1987) using ABTS as a substrate. FDH activity was assayed by a continuous spectrophotometric rate method according to Hopner and Knappe (1974) following the consumption of \(\beta\)-NAD as a cofactor. Cell dry weight (CDW) was measured by sampling 5 mL of culture, centrifuging for cell separation and drying the pellet at 70 °C.

![Graph](Image)

Figure 1 shows the acquired results from the experiments. As this figure presents, from an initial value of 0.61 g/L, CDW reached to 26.31, 56.49, and 95.86 g/L at the end of glycerol batch, glycerol fed-batch, and methanol fed-batch phases, respectively. There was an obvious coordination among the activities of AOX, FDH and \(\beta\)-galactosidase. All of these enzymes reach their maximum specific activity approximately 12 hours after methanol fed-batch phase, which is also the beginning of the induction for the production of these enzymes, and then their activity declines gradually. Attaining the maximum specific activity of \(\beta\)-galactosidase after 12 hours of induction suggests that only 12 hours of methanol feeding in methanol fed-batch phase would be enough for obtaining maximum possible enzyme activity per biomass unit and so the amount of biomass at the induction time may play an important role. In other words, biomass may be increased much more in the glycerol fed-batch phase, which may reduce the total fermentation time and achieving higher \(\beta\)-galactosidase activities in shorter time. The simultaneous reduction in specific enzyme activities is due to the toxic effects associated with the accumulation of formaldehyde in culture, which had induced FDH activity, and reduction in the methanol consumption capacity of the cells.

Keywords \textit{Pichia pastoris}; alcohol oxidase; formate dehydrogenase; \(\beta\)-galactosidase; fed-batch.
Salmonella enterica Typhimurium: Establishment of cultivation condition on shake flask and flagellin purification strategy by using tangential ultrafiltration.

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Flagellin is a main protein present in the bacterial flagellum and its molecular mass is around 51.5 kDa. Its role as an adjuvant vaccine has been intensively studied and currently there is evidence to be an immunomodulator in situations of autoimmune diseases, co-administered with chemotherapy or as radioprotector. This study focuses to establish the native flagellin production process produced by Salmonella Typhimurium in shake flask and flagellin purification strategy by using tangential ultrafiltration. Some parameters were studied in order to extract the flagellin from the cell and/or release to the culture. Results showed that flagellin can be released to the supernatant depend on the agitation speed and tangential ultrafiltration can be used as tool to purify this protein.

Keywords: Salmonella, flagellin, homogenizer, tangential ultrafiltration.

Scale-up of a Solid-State Bioconversion Process for Lovastatin Production in a 1200 Liter Reactor

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The process for lovastatin production by solid-state bioconversion using an Aspergillus sp. was scaled up from 7 L to 1200 L. It was demonstrated that by maintaining a constant superficial airflow velocity through the bed of solid substrates at these two scales of operation results in similar productivity of lovastatin. The experiments in the 1200 L reactor were carried out according to a central composite design using the airflow rate and substrate composition as variables. The performance in the different experiments was evaluated based on the lovastatin production. From a statistical analysis of the results, it was determined that the desirable operating condition for maximum lovastatin production was 22 cm min⁻¹ superficial airflow velocity and 53% of wheat bran in solid wheat straw substrate. The maximumLovastatin production of 2.14 mg g⁻¹ (dry substrate) was obtained in the 1200 L reactor compared to 2.68 mg g⁻¹ (dry substrate) obtained in the 7 L reactor.

Keywords: Scale-up, superficial airflow velocity, solid-state bioconversion, lovastatin production, Aspergillus sp.
Screening and characterization of lactic acid bacteria for the production of mannitol in carob based syrups

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Mannitol, a naturally occurring polyol, is widely used in medicine, and in pharmaceutical, food, and chemical industries. It can be produced by chemical, microbiological or enzymatic processes. The industrial chemical production is based on the hydrogenation of fructose/glucose mixtures at high temperature and pressure using Raney-nickel catalyst yielding a mixture of mannitol and sorbitol, an isomer, which has less interesting properties and hence a significantly lower market price. Moreover, the separation of mannitol and sorbitol is rather difficult. The enzymatic process requires the use of redox co-factors, which renders it unattractive. Because of these problems, mannitol production by the microbial route has become attractive, even at the industrial scale. Heterofermentative lactic acid bacteria (LAB) are among the most efficient for mannitol production, using glucose/fructose mixtures without producing sorbitol which makes unnecessary purified substrates or complex processes for product purification. Carob pulp is a by-product of the carob gum industry with high sugars content namely sucrose (the main sugar), glucose and fructose that can be used as a cheap source to easily obtain sugar-rich syrups.

In this work, a screening program was developed to identify mannitol overproducing LAB in carob based media. First, the sugar extraction from carob pulp kibbles was optimized. The optimal conditions were defined for a liquid-to-solid ratio of 2 kg water/kg kibbles, temperature 50°C for 5 h. In order to hydrolyse sucrose, a subsequent mild dilute acid hydrolysis, using sulphuric or hydrochloric acids was also studied. The hydrolysates contained, on average, 110 g/l fructose and 95 g/l glucose without significant amounts of microbial inhibitors, namely 5-hydroxymethylfurfural that did not exceed 0.6 g/l. The screening conditions were then defined using both Lactobacillus fermentum ATCC 9338 and Weissella confusa DSM 20916 as model microorganisms. The screening was performed in semi-aerobic conditions at 30°C or 37°C, depending on the optimum growth temperature for each strain.

Thirty bacterial strains of the genus Lactobacillus, Leuconostoc and Weissella, obtained from several cultures collections and laboratory isolates were tested. From these, a restricted group of eight strains were identified as potential mannitol overproducers, among which Leuconostoc genus was significantly represented. The most promising strains were Lc. citreum ATCC 49370, Lc. mesenteroides subsp. cremoris ATCC19254, Lc. mesenteroides subsp. dextranicum ATCC 19255, Lc. casei ATCC B-23447, Lc. casei ATCC B-23447, Lc. casei ATCC 19256, L. intermedes NRRL 3692 and L. reuteri DSM 20016.

Keywords: carob, lactic acid bacteria, Lactobacillus, Leuconostoc, mannitol production

This work was supported by Adl, Project ValorAlfa (70/00326). Joana Pereira gratefully acknowledges a grant from Adl.

ARS Culture Collection (National Centre for Agricultural Utilization Research, Peoria, IL, USA) and Dr. Isabel Fernandes (INETI-DTIA, Lisboa, Portugal) are gratefully acknowledged for supplying bacterial strains.

Screening for antibiotics from indigenous Streptomyces, their genetic and mutational analysis

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110 active Streptomyces strains were selected from the soil samples collected from different sites of the province Punjab, Pakistan. The isolated strains were characterized morphologically, biochemically and physiologically, in 16s rRNA gene sequence analysis the strains exhibited genetic similarity with different Streptomyces species e.g. Streptomyces chromofuscus, Streptomyces macrosporeus, Streptomyces matensis, Streptomyces vinaceus, Streptomyces griseooccinarius, Streptomyces malachitofuscus etc. The strains exhibited promising antimicrobial activity against a variety of indicator microorganisms including Staphylococcus aureus, Bacillus subtilis, E. coli, Candida albicans, Mucor miehei, Chlorella sorokiniana, Chlorella vulgaris and Scenedesmus subspicatus and cytotoxicity against Artemia salina in biological screening and an impressive metabolic diversity, in chemical screening by TLC and HPLC-MS analysis. In preparative screening the selected isolates were cultivated in a 50 liter lab fermenter and more than 30 compounds were isolated, purified and identified from the culture broths of the six strains by MS and NMR analysis. Almost all the structural classes of the bioactive metabolites were found to be produced by these indigenous Streptomyces including, cyclic thiopeptides (geninthiocin and the new val-geninthiocin), macrolides (chalcomycin, Emycin D), polyether (alborexin), perhydroxy quoinones (ochromycinone,) along with actinomycin D, resistomycin and tetracycin D etc. In mutational studies with one of the strain producing new cyclic thiopeptide (val-geninthiocin), mutants with improved production ability were selected. The study reveals that the indigenous Streptomyces flora is a potential source of interesting antimicrobial agents and its continuous exploration may yield novel metabolites which can be helpful to combat the uprising problem of antibiotic resistance among the pathogenic species.

Keywords: Indigenous Streptomyces, Antibiotics Screening, Val-geninthiocin, Mutational Analysis.
Searching for proteins that influence biotransformation of VitaminD₃ in Rhodococcus erythropolis.

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Vitamin D₃ (VD₃) is a steroid hormone that plays crucial roles in bone metabolism, immunity, and control of cell proliferation and cell differentiation in mammals. VD₃ is inert and must be activated to exert its biological activity. We have recently identified new cytochrome P450 named Vdh from the actinomycete Pseudonocardia autotrophica, which is capable of biocconversion of VD₃ into its physiologically active forms of VD₃, 25(OH)VD₃ or 1α,25(OH)₂VD₃. Biotransformation of VD₃ into 25(OH)VD₃ is accomplished with a recombinant strain of the actinomycete Rhodococcus erythropolis coexpressing Vdh and reductase proteins, ferredoxin and ferredoxin reductase. To characterize the mode of biotransformation of VD₃ into hydroxylated forms of VD₃ in vivo, we constructed transposon mutant library of the recombinant R. erythropolis and screened mutants with no biocconversion activity of VD₃. For this purpose, we established a high-throughput assay system by using yeast two-hybrid system for detection of 25(OH)VD₃. Over 40,000 mutants were screened and 12 mutants, which showed a little or no biotransformation activities, were isolated. The insertion site of transposable marker in each mutant was analyzed. The sequence analysis of the insertion sites revealed that the transposable maker gene was inserted in genes encoding proteins annotated as an unknown function, membrane protein transcription factor, etc. Functional characterization of each gene is on going and the data of ferredoxin deficient mutant will be presented.

Keywords Vitamin D₃, Vdh; Vitamin D₃ hydroxylase, Biotransformation, Two-hybrid assay, Rhodococcus erythropolis

Separation of catalytically active enzymes through foam fractionation

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Numerous enzymes for the food, pharmaceutical and cosmetic industry are currently isolated by multistep processes, which are generally time-consuming, expensive, and above all, often come along with high losses of enzymatic activity. Foam fractionation, which belongs to the group of adsorptive bubble separation techniques, represents a gentle and inexpensive alternative downstream process for the selective isolation of extracellular enzymes from microbial culture media. The method is based on the adsorption of surface-active compounds to the interface of a gas-liquid dispersion, and it of a gas-liquid dispersion, and it is especially suitable for the enrichment of enzymes from diluted solutions, as they typically occur in biotechnological processes. While gas bubbles rise through a liquid phase which contains the target solute, the most surface-active molecules preferentially adsorb to the interfacial area. The dynamics of the adsorption are affected by a number of operational parameters such as pH value, temperature, and superficial velocity.

Laccases, which currently gain a great deal of attention for bio remediation, beverage processing and baking, were produced in submerged cultures of the edible basidiomycetous fungus Pleurotus sapidus. The secretion of laccases into the culture media was increased significantly by the addition of specific enzyme inducers. After optimisation of the physico-chemical parameters of the foaming process and after addition of the cationic detergent CTAB (cetyl trimethyl ammonium bromide) to the culture supernatant, up to 70% of the total laccase activity was transferred into the foam. Depending on their respective isoelectric points a fractionated transport of five laccase isoenzymes was achieved by variation of the pH, and one isoenzyme was transferred into the foam phase with a recovery of activity of 100%

The secretion of two lipases/esterases by P. sapidus was induced by supplementing the culture broth with Tween 80, which also served as a foaming agent. After foaming, up to 95% of activity was recovered in the foam; the removal of the mycelium pellets from the culture supernatant prior to foam separation was not necessary. Modifications of the foaming device increased the drainage effect and resulted in superior enzyme recoveries when compared to traditional multistep procedures. Differences in their physicochemical characteristics resulted in differing foaming properties of the two lipases/esterases secreted by P. sapidus. By variation of the pH value and of the addition of detergent, both lipases/esterases were successively and quantitatively transferred into the foam in a two-step fractionation process. On a pilot plant scale, quantitative recoveries of activity were obtained documenting the feasibility of the implementation of foam separation into industrial enzyme purification strategies.

Keywords Pleurotus sapidus, Laccase; Lipase; Esterase; Foam Fractionation; Enzyme Purification; Adsorptive Bubble Separation
Strains selection on aliphatic substrates: first step for omega-oxidation of C9-C18 fatty acids to obtain biopolymers from waste.

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Biodegradable polymers find application in a number of commercial products, so-called “environmental friendly”. To overcome limitations of synthetic polyesters, biodegradable polyesters may be obtained from vegetable oils, a renewable source of fatty acids (FA) representing an interesting substrate thanks to their large availability, low cost, and biodegradability. The limiting step of FA polycondensation is the lack of a second carboxyl or hydroxyl function in a terminal (ω) or subterminal (ω-1) position. Chemical processes capable to produce dicarboxylic or hydroxyl acids starting from FA already exist, nevertheless all these processes have intrinsic drawbacks. As a consequence, new oxidative processes are needed, able to introducing a second chemical function besides the carboxyl group in natural FA.

The purpose of this study is to select microorganisms capable of oxidize FA in ω position and to maximize this reaction minimizing β-oxidation.

We selected two strains able to grow on alkanes (nonane, decane, dodecane, hexadecane and octadecane) as sole carbon source: the ability of metabolize these substrates suggests the presence of an ω-oxidative enzyme system. The first strain isolated is Acinetobacter radioresistens S13, a Gram negative bacterium able to hydroxylate aromatic compounds (phenol, benzoate) and to grow on surfactants like Tween 40, 60, 80 (Pessione et al. 1997).

The second strain is a Gram positive bacillus, now under identification. It was selected from a freeze-dried obtained from the soil surrounding an activated sludge pilot plant.

For these two strains we also tested the growth on alkanes with supplementation of surfactants to increase substrates bioavailability, and growth and toxicity of several C6-C18 fatty acids.

Experiments are underway to inhibit catalitically, or by gene deletion, β-oxidative enzymes in order to obtain ω-hydroxy and ω-carboxy fatty acid by co-metabolism with a second low-cost carbon source.

Keywords: alkanes; omega-oxidation.

Studies on the of specificity of some lipase-catalysed hydrolysis and esterification reactions

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In the last few years, there has been an increase in the use of naturally derived compounds, such as essential oils, that justifies the increase observed in oils production. Biotransformation of vegetable oils through the use of enzymes as catalysts has been also a matter of intense investigation nowadays.

In this study four enzymes (three commercial lipases: Lipozyme TL100L, Resinase HT, Resinase A2X) and lipase from Enterobacter aerogenes 13 (EAL, JSC Biocentras, Vilnius) were compared for their potential and specificity in hydrolysis reactions of nine different oils (rapeseed oil (RSO), false flax (Camelina sativa) oil (FFO), sunflower oil, olive oil, castor oil, cocoa butter, linseed oil, rice bran oil and Hyssopus officinalis L. essential oil). Hyssopus officinalis L. plants were collected near Vilnius (Vilnius district, Lithuania). Essential oils from six localities obtained by hydrodistillation were analysed using GC and GC/MS.

The work had the objective to study the effect of temperature and reaction time on the formation of free fatty acids from different oils through lipase-catalyzed oil hydrolysis. Comparison between thin layer chromatography (TLC) supplemented with pixel area scanning technique using total Lab Program version Microimage 4 for the measurement of remaining triglycerides GC and free fatty acids, GC/MS and titrimetric methods was done to determine the optimum process conditions.

It was shown that EAL lipase-catalyzed hydrolysis reactions were the most effective at 30°C, while commercial lipases were more active at 50°C.

The ability of soluble and immobilized lipase from EAL to catalyze transesterification of rapeseed oil (RSO) with methyl and ethyl alcohols and also esterification of different fatty acids with several alcohols was investigated first time. The work had three objectives: 1) to study the effect of temperature, solvents, chain length of fatty acids, reaction time and the quantity of lipase and immobilization on the formation of free fatty acids from two different oils through lipase-catalyzed hydrolysis; 2) to study the effect of temperature, the quantity of water and lipase and method of enzyme immobilization on the formation of fatty acid alky esters from RSO through lipase-catalyzed transesterification reactions; 3) to study the effect of different alcohols, the chain length and saturation of the fatty acids and the quantity of the enzyme on the formation of fatty acid alkyl esters from various fatty acids and alcohols through lipase-catalyzed esterification reactions using thin layer chromatography and titrimetric methods.

Keywords: Enterobacter aerogenes lipases; oils; hydrolysis; transesterification; esterification. Acknowledgments: The Lithuanian State Science and Studies Foundation (Contracts No. N-01/2009 and N-10/2009) is gratefully acknowledged for financial support.
Study effect of antimicrobial Chitosan and Cellulase enzyme on against spoilage microorganism in date syrup in HACCP system

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Introduction and Object: Date syrup is a highly nutritious food product, rich in calories and many vitamins and minerals that produce composition date tamer and water whit heat and that quality decrees during act of bacterial and fungus microorganisms. The object of this research was to investigate use Chitosan and Cellulase enzyme instead of chemical filtration in industrial method on taste quality and suitable color, stability and quality product and rate growth of microorganism.

Material and Method: The effects of chitosan and Cellulase against food spoilage microorganism in date syrup were investigated with using method of Institute of Standard and Industrial Research of Iran.

Results: HACCP is a food safety inspection system. After that the Critical Control Points (CCP) were knowing in the proceeding of date syrup , single Cellulase showed no effect on microorganism in this CCP, and when chitosan used 2,4,6% concentration , with increasing amount of Chitosan it showed decrees in number of spoilage microorganism. Finally when Chitosan and Cellulase used together showed significance decreasing in rate of growth in microorganisms differences when they used separate.

Conclusion: The high molecular weight of Chitosan, which result in a poor solubility at neutral pH values and high viscosity aqueous solutions, limits its potential uses in the fields of food, health and agriculture, most of these limitations are overcome by Chitosan oligosaccharides obtained by enzymatic hydrolysis of the polymer such as Cellulase.

Keyword: HACCP, Chitosan, Cellulase, Date syrup.

Synthesis of cellulases and xylanases from mutant PR-22 of Cellulomonas flavigena under catabolic repression conditions.

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Lignocellulosic biomass is the most abundant carbon source on Earth and represents the major source of renewable energy. Sugarcane bagasse is specifically attractive because of its low cost and plentiful supply. The complete saccharification of lignocellulosic biomass for ethanol production can be achieved using cellulases and xylanases. The cost of enzyme production is one of the factors that determine the viability of the bioprocess, thus the design of highly efficient enzymes and finding hyper-producing mutants could reduce these limitations, increasing the economic feasibility. Synthesis and activity of cellulases and xylanases are regulated by induction, repression and inhibition phenomena. Mutant isolation, with a proper screening and selection strategy, is an excellent method to get derepressed strains. The treatment of C. flavigena PN-120 with N-methyl-N´-nitro-N-nitrosoguanidine (NTG; 150 μg·mL⁻¹) and 2-deoxiglucose (2DG; 0.5%) as selection strategy allowed obtaining mutant PR-22, a derepressed hyperproducer strain.

The aim of this work was to analyze the behaviour of carbomethyl-cellulase (CMCase) and xylanase activities under catabolic repression and inhibition conditions and the improvement achieved in PR-22 strain growing in sugarcane bagasse.

Mutant PR-22 exhibited higher specific enzyme activities than parent strain PN-120 and had an improvement index (IMI) of 32 and 190% in xylanase and CMCase activities respectively. Several concentrations of glucose or cellobiose, as repression and inhibition conditions were added to cultures of C. flavigena PN-120 and PR-22 previously induced with sugarcane bagasse in batch culture. Inhibition index (II) was estimated on basis of activity showed by each strain respect to that showed growing without glucose or cellobiose (controls). Glucose at 10 mM did not repress the enzyme production of mutant PR-22 and this strain showed a slight II of -3% only in xylanase activity. In same conditions, strain PN-120, showed IIs of -9 and -20% on xylanase and CMCase activities. When 20 mM of glucose was added, soluble protein was still produced in both strains while CMCase and xylanase activities had IIs of -28 and -41% for PR-22 and -32 and -49 % for PN-120 strain respectively.

Cellobiose experiments had higher IIs in both mutants. However mutant PR-22 was more resistant to cellobiose than its parent strain PN-120. Cellobiose at 20 mM had IIs of -45 and -31 % over xylanase and CMCase activities in PR-22 while in PN-120 these activities had IIs of -41 and -45% respectively. In vitro studies, the enzymatic complex of PR-22 showed more resistance to inhibition than enzymes from PN-120 strain. Saccharification studies of sugarcane bagasse (3 %) with crude enzyme extract from PR-22 yielded at least 10 g·L⁻¹ of reducing sugar equivalents. Mutant PR-22 of C. flavigena has characteristics and behavior of a derepressed mutant since glucose 20 mM did not affect the production and activity of cellulolytic enzymes. C. flavigena wt repressed completely its holocellulosic enzymatic system at 10 mM glucose (Ponce-Noyola & de la Torre, 2001). PR-22 strain is a good candidate to be used for producing cellulases and xylanases in a large-scale bioreactor and its later use in lignocellulosic biomass saccharification.

Keywords Cellulomonas; cellulase; derepressed; hyperproducer
The application of PCR methods and gas chromatography for detection of specific non-pathogenic bacteria of the genus Clostridium

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The aim of this study was to select and investigate non-pathogenic bacterial strains of the genus Clostridium collected in the Dairy Research Institute. The strains were isolated from cheeses with late blowing disorders. First, a set of 44 strains was identified into the genus Clostridium using genus-specific PCR (Rekha et al., 2006). Purified DNA (phenol extraction) was used as DNA matrix. PCRs specific to the species Clostridium butyricum (Nakanishi et al., 2005) and Clostridium tyrobutyricum (Herman et al., 1995) were used for species identification. It was found that 7 and 12 strains belonged to the species C. butyricum or C. tyrobutyricum, respectively. Some strains were not identified into species yet. Strains encoding the H2-evolving hydrogenase (hydA) gene were also identified using PCR (1). Specific PCR products were detected in 30 strains (70%) of the analysed set. Formation of fatty acids (acetic, propionic, and butyric) was studied by gas chromatography in culture supernatants. The molecular parameters (data) received will be used for correlation with hydrogen production.

References

Keywords: Clostridium butyricum; Clostridium tyrobutyricum; PCRs; hydrogenase (hydA); gas chromatography

The production of volatile compounds by yeasts isolated from artisanal brazilian cachaça distilleries

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Thirty yeast strains were evaluated with respect to their fermentative characteristics for the production of cachaça, twenty-four being Saccharomyces cerevisiae strains and six belonging to the following genera: Candida (3), Kloeckera, Pichia and Schizosaccharomyces. They were isolated from small cachaça distilleries (27), industrial cachaça distilleries (2) and one sugarcane alcohol distillery. They were evaluated with respect to the main volatile compounds produced in a synthetic media. The compounds analyzed were acetaldehyde, ethyl acetate, propanol, isobutanol, isoamyl alcohol, acetic acid and glycerol. The Saccharomyces strains showed a limited variation of about 50% of the average rate, with respect to the rate of production of each volatile compound. The Hierarchical Cluster Analysis and the Principal Component Analysis showed the separation of the strains into several Groups, the rate of acetic acid production being the variable of greatest impact in the differentiation of the strains. The strains of S. pombe formed a distinct group (Group 2), and the strains of C. apicola and H. occidentalis formed a joint group (Group 6) as did Sc13 and Sc4 (Group 4). Group 1 was formed exclusively of S. cerevisiae. The closest non-Saccharomyces strains were C. apicola and H. occidentalis, with a similarity index of about 0.95. The strains P. subpellucida showed general characteristics more similar to those of the S. cerevisiae strains than to the non-Saccharomyces strains.

Key Words: cachaça, yeast, Saccharomyces cerevisiae, volatile compounds, alcoholic fermentation.

Acknowledgements: We thank Fundação de Amparo à Pesquisa de Minas Gerais (FAPEMIG) for financial support.
The specificity of an Enterobacter aerogenes 13 lipase

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The increasing interest for lipases as one of the best biocatalysts for the catalysis in organic media caused the task to investigate the esterification ability of the lipase. The esterification reaction of oleic and erucic acids with various alcohols catalyzed by selected soluble lipases including Enterobacter aerogenes 13 (EAL, I3C Biocentrus, Vilnius) and Pseudomonas mendocina 3121-1 lipase (Institute of Biochemistry, Vilnius) and Lipoprime 50 T was investigated.

It was shown that lipolytic enzyme produced by EAL was active when catalyzing the esterification reaction of oleic acid with ethanol, ethane 1,2-diol (ethylene glycol), propane 1,2-diol (propylene glycol) and 2-hydroxyethyl ether (diethylene glycol). The determination of kinetic parameters of Pseudomonas mendocina 3121-1 lipase for different p-NPR esters in 2-propanol showed that K_m was highest for esters of short chain and lowest for p-nitrophenylcaprylate (p-NPC), i.e. the substrate of moderate chain. So it could be concluded that P. mendocina lipase is specific for p-NPR esters of moderate and long chain and the most specific for p-NPC. The highest V_max for p-NP-laurate and p-NP-myristate also indicated the most effective hydrolysis of substrates of moderate chain. V_max/K_m (calculated ratio) for p-NPR esters of moderate and long chain far exceeded the ratio for p-NP-acetate and p-NP-butyrate.

The esterification reaction of erucic acid with various alcohols (C3-C18:1) was also investigated and it was found that Enterobacter aerogenes 13 lipase showed the highest specificity to long-chain alcohols while Lipoprime 50T was the most active when esterifying short-chain (C3-C8) alcohols and Pseudomonas mendocina 3121-1 lipase esterified catalyzed esterification of medium-chain alcohols most effectively, particularly octanol. Thin layer chromatography (TLC) and titrimetric methods were used to determine the optimum process conditions. Quantitative analysis (%) of reaction products separated by TLC was performed using the micro image 4.0 program supplemented with pixel area scanning technique.

The ability of EAL to catalyze hydrolysis of different oils and also transesterification of rapeseed oil (RSD) with methanol and ethanol was investigated first. The optimum condition for enzymatic hydrolysis of RSO was found to be at 50°C with the highest quantity of enzyme used in non-aqueous media, and at 30°C with the lowest quantity of enzyme in aqueous media. For enzymatic hydrolysis of false flax (Camelina sativa) oil (FFO) it was found to be at 50°C with the highest quantity of enzyme in both, non-aqueous and aqueous media. FFO hydrolysis also was found to be more effective than RSO hydrolysis for both, native and immobilized enzymes. It was shown that EAL catalyzed reaction is the most effective at room temperature, while Resinaze HT and Lipolase are more active at 50°C. The detection of increasing amount of free FA was determined both by titrimetric method and by TLC.

Keywords: Enterobacter aerogenes 13 lipase; specificity; fatty acids; p-NP esters; esterification

The Lithuanian State Science and Studies Foundation (Contracts No. N-01/2009 and N-10/2009) is gratefully acknowledged for financial support.

Towards a consolidate bio-processing for the conversion of agroindustrial wastes into optically pure lactic acid

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The demand of lactic acid (LA) has been considerably increasing recently, owing to the promising applications of its polymer, the polylactic acid or polylactide (PLA), as an environment-friendly alternative to plastics derived from petrochemicals. High biodegradability and biocompatibility of such materials, render them particularly suitable for surgical (e.g. orthopaedic, cardiovascular and sutures) and other medical (dialysis and drug delivery devices) applications. For synthesizing polylactides with adequate physical properties, it is essential to polymerize optically pure LA: the best way to obtain optically pure LA is by microbial fermentation, while chemical synthesis always results in a racemic mixture of LA.

Our present research projects are aimed to set up a consolidate bio-processing(s) for the conversion of agroindustrial (fruit and vegetable wastes, lignocellulosic biomasses, milk whey) wastes into optically pure LA. Since lactic acid bacteria (LAB) are among the chief sources of lactic acid, LAB strains able to metabolize the most abundant soluble carbohydrates (fructose, xylose, cellubiose, lactose) present in different agroindustrial wastes were selected from the microbial collection of our laboratory. By acting on the fermentation parameters (e.g. pH, pO2 and acetate supplementation) the enantioselectivity of the process could be increased, leading to the production of L-LA mainly.

Unfortunately, natural LAB strains are not able to degrade cellulose. Therefore, a metabolic engineering strategy is now applied to construct a cellulosolytic LAB suitable for the bio-conversion of cellulotic biomasses. Two Lactococcus lactis strains have been selected for their ability to catabolize both xylose (the main constituent of hemicelluloses) and cellubiose (the repeating unit of cellulose) besides glucose. Heterologous expression of the main components of the cellulosome (the protein complex responsible for cellulose hydrolysis) of Clostridium cellulovorans in L. lactis is currently underway. The aim is to express a minicellulosome containing the minimum number of components needed for a functional cellulase system.

Keywords: LAB, lactic acid, metabolic engineering, minicellulosome, agroindustrial wastes; consolidate bio-processing.


Keywords: LAB, lactic acid, metabolic engineering, minicellulosome, agroindustrial wastes; consolidate bio-processing.
Use of the branched-chain amino acid biosynthetic pathway for conversion of sugars into higher alcohols

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Production of biofuel from renewable sources is now of particular interest due to the limited reserves of traditional energy sources such as fossil fuels, unstable oil prices, and the necessity to limit greenhouse gas emissions and to reduce air and water pollution. Plant biomass is one of the most important renewable energy resources. Important steps have been made to increase biomass utilization for production of hexose and pentose and also to adapt microorganisms for the simultaneous consumption of a variety of carbon sources. Conversion of C5, C6 sugars into liquid energy carriers occurs in two major anaerobic biological processes: ethanol fermentation and a mixed acetone, ethanol and butanol fermentation. Currently, the biosynthesis of higher alcohols (butanol, isobutanol, 2-methyl-1-butanol, etc.) from plant biomass has gained significant attention due to advantages over ethanol with respect to use as motor fuels. In recent years, several research groups have proposed the use of highly effective amino acid biosynthesis pathways as an alternative approach for aerobic production of the higher alcohols as new-generation biofuels (Donaldson G.K. et al., 2007; Liao J.C. et al., 2008, 2009). Here, using E. coli as a model, we evaluate the possibility of using pathways of branched chain amino acid biosynthesis for aerobic sugar conversion into higher alcohols.

To redirect the branched-chain amino acid biosynthesis into production of higher alcohols (particularly isobutanol), we attempted to use the Ehrlich pathway to convert ketoisovalerate, the keto-precursor of valine, into the corresponding aldehyde and subsequently into isobutanol using two enzymes: branched-chain α-keto acid decarboxylase (BCKAD) and alcohol dehydrogenase (ADH). As E. coli has no enzymes for this purpose, the BCKAD-encoding gene kdcA of Lactococcus lactis and the ADH-encoding gene adh2 of Scecharomyces cerevisiae. The gene kdcA L. la contained a number of codons rare for E. coli. To ensure a high level of BCKAD expression, the synthesis of a modified kdcA gene with rare codons substitutions was therefore carried out, and the gene adh2 S ce was cloned in its native form. Both of these genes were cloned in a low copy number vector under control of the lactose operon promoter, thereby generating the artificial expression unit P goals-kdcA-adh2. The resulting plasmid pMW118-kdcA- adh2 was introduced into the valine-producing strain H-81 (VKPM B-8066). Induction of the Ppara-kdcA-adh2 operon by addition of IPTG into the cultivation medium resulted in a 6-fold decrease of Val accumulation in comparison to the H-81 strain lacking plasmids and, in addition, in poor growth of cells. The plasmid strain accumulated approximately 1.1 g/l of isobutanol under IPTG induction in test tube cultivation in the medium containing 6% of glucose. For the strain H-81 that lacked plasmids, no isobutanol accumulation was detected, indicating the absence of native E. coli enzyme systems of ketoisovalerate degradation via the Ehrlich pathway under aerobic conditions. The plasmid pMW118-kdcA containing only P para-kdcA without the adh2 gene was introduced into the strain H-81. It is worth noting that the possession of BCKAD alone also resulted in isobutanol accumulation in culture broth (up to 0.34 g/l). This fact indicated that native alcohol dehydrogenases of E. coli were responsible for isobutanol production, but with less efficiency than the Adh2 of S. ce. It is necessary to note that, under tested aerobic cultivation conditions (68 h, 32°C), a significant portion of isobutanol was experimentally confirmed to evaporate. Measurement of the activity of L. la BCKAD revealed that this enzyme was about 3.6 times more active in relation to ketomethylvalerate when compared to ketoisovalerate; this indicated that it was possible to produce 2-methyl-1-butanol using this enzyme.

Using E. coli as a model, the aerobic synthesis of isobutanol from glucose was achieved by means of the host enzymes in branched-chain amino acid biosynthetic pathways together with heterologous Ehrlich pathway enzyme systems. Considering the recent significant progress in the development of membrane technologies of higher alcohol separation and the promising results of the screening and characterization of alcohol-tolerant species, further engineering of microorganisms for effective conversion of biomass-derived sugars into fuel alcohols can create a basis for implementation of this approach into the production of biotechnological fuels.

Keywords: fusel alcohols; isobutanol; biofuel; branched chain amino acids
A chemiformatics approach to produce an enriched database of antitubercular compounds useful for drug discovery pipeline

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Tuberculosis (TB), a disease caused by the facultative intracellular bacterium Mycobacterium tuberculosis (mtb), is a global health problem and a leading cause of death among adults in the developing world. In 2006, there were approximately 9 million new cases of TB, resulting in an estimated 1.7 million deaths. Although it is primarily a scourge of the developing world, tuberculosis affects virtually every nation and every ethnicity. Within this epidemic, the incidence of Multi-Drug Resistant tuberculosis (MDR-TB) appears to be rising with previous estimates being a gross underestimate as revealed in a recent 2008 report. The emergence of MDR Mycobacterium tuberculosis strains, the growing rate of TB incidence, the lethal combination represented by HIV co-infection and the lack of any new antituberculosis agent in the last 40 years, which are based on agents developed in the 60-80s, all indicate an urgent need for the development of novel TB therapeutics. In particular, new lead structures are required with novel modes of action.

To obtain new chemical entities capable of fighting the causing agent of TB, the sources are natural products and new compound synthesis. The rational on selecting the substances are random screening, traditional medicine, Structure-Activity Relationships (SAR) for synthetic compounds, and computer-aided discovery programs or in silico methods. One of the main components in an in silico application is the design of applicable and new compound library or database which accordingly can help in setting the virtual boundaries for selecting rational design. In this study we describe how such useful database was established.

At first a fragment library, with total of 50 classes, was formed after ontology-based classification of about 2789 non-redundant compounds by Library MCS, 0.6, 2008, obtained from Enhanced NCI using the passpredict for antitubercular property. The fragments were subjected to similarity search in Pubchem with a 70% similarity cutoff to include commercially available brand named compounds into the database forming sdf format compatible for 3D search and library docking. The library of Merck catalogue was searched for a fragment-based pattern similarity to add further set of about 23 to already found non-redundant set of 283 compounds containing the fragments obtained in Enhanced NCI group of compounds. The compounds were filtered for attempted compounds (1117 entries) with positive antitubercular bioassay results (166) and they were excluded from library using ChemFinderUltra, 9.0, 2005.

Finally, after removing all duplicate molecules, the resulting database contained about 3037 compounds, including the fragments of antitubercularly prone substances. This database can be used for synthetic drug development approach as it includes the high indexed commercially available molecules and fragments. There are also applications for template similarity tagging which can be made in the discovery mode for anti-tubercular pre-screening studies.

Keywords: in silico method; database; antitubercular; compound

A critical view on a theory coupling cell growth and DNA replication initiation through individual-based modelling

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Introduction and objective
The mechanisms and regulation of DNA replication initiation in bacteria are not yet fully understood (for reviews, see e.g., Haeusser and Levin (2008), and Mott and Berger (2007)). One of the most challenging parts seems to be the coupling of cell growth to DNA replication initiation. For over four decades, the dogma has held that the initiation of replication is triggered at a specific cell size, which is independent of growth rate (Donachie, 1968). More recently, Donachie and Blackley (2003) have proposed a hypothesis incorporating available individual mechanistic knowledge about the initiator protein DnaA linking cell size to DnaA dynamics. In this theory, DNA replication is initiated whenever the ratio DnaA-ATP over DnaA-ADP reaches a critical (or specific) value. In this study, the hypothesis of Donachie and Blackley (2003) is mathematically translated into and tested in an individual-based modelling environment.

Individual-based modelling considers the individual cell as the modelling unit and emergence of population dynamics is investigated via the fundamental unit of bacterial life, an individual cell. Each individual is considered as an independent entity with its own state (e.g., mass, age, ...) and behaviour. Nevertheless, all individuals are of the same type (e.g., bacterium) and have the same potential regarding state and behaviour. As individual microbial behaviour is translated into rules and/or equations, BiM provides a perfect framework to test hypotheses concerning microbial behaviour. Individual and emerging population dynamics can then be validated against experimental data and prior knowledge.

Results
Different aspects of the theory are investigated. To exclude anomalies originating from stochastic events, individual variability is not included in a first phase. A link between the critical ratio and cell mass is proposed. The influence of the growth rate and the critical ratio on both individual and population (mass and DnaA) dynamics is investigated. Under precise conditions, individual and population dynamics display normal behaviour and the bacterial cells accurately double during a generation. Deviation from these precise conditions leads to anomalies (or unrealistic results) in the microbial behaviour, e.g., increase/decrease of cell over generations, and accumulation of DnaA.

Conclusions
Critical appraisal of hypotheses concerning microbial behaviour through individual-based modelling provides insight in individual and emerging population dynamics. Although the theory of Donachie and Blackley (2003) combines previously opposing theories and offers valuable insights, additional mechanisms need to be incorporated to ensure cell size doubling, accurate onset and timely completion of DNA replication initiation.

Acknowledgements
Work supported in part by Projects OT/09/25 and EF/05/006 (OPTIEC Optimization in Engineering) of the Research Council of the Katholieke Universiteit Leuven, and by the Belgian Program on Interuniversity Poles of Attraction, initiated by the Belgian Federal Science Policy Office. K. Bernaerts is a Postdoctoral Fellow with the Fund for Scientific Research Flanders (FWO-Vlaanderen). The scientific responsibility is assumed by its authors.

References

Keywords: individual-based modelling, DNA replication initiation, predictive modelling
A Lovastatin Production Model Possessing Features for Process Control Applications

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Lovastatin is produced industrially by a variety of filamentous fungi. It is a potent drug for reducing blood cholesterol and acts by competitively inhibiting 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase). The productivity of lovastatin in submerged cultivation is affected by the medium composition, oxygen mass transfer and conditions of reactor operation. The productivity of lovastatin can be significantly improved if an advanced control strategy is implemented. This turn depends on the development of a model that satisfies the structural properties of nonlinear observability and controllability. In this paper, the development of one such model employing biomass, glucose, lactose, glutamate, lovastatin and dissolved oxygen concentrations as state variables is presented. The process evolution can be directed by varying the nutrient feed rate and airflow rate. The model was derived and its parameters identified from experimental data obtained from batch experiments carried out using Aspergillus sp. in a 15 L bioreactor. The complete systems theory analysis was performed to establish that the model was suitable for online process control applications. As an example, a nonlinear input-output linearizing controller was derived and a stability analysis was performed to demonstrate that the internal variables were bounded.

Figure 1. The state-time profiles biomass, glucose, lactose, Na-glutamate, lovastatin and dissolved oxygen concentrations obtained from a 15 L batch experiment.

Keywords: Lovastatin model, submerged fermentation, nonlinear structure; systems analysis; Aspergillus sp.

Advances in detection of inorganic pollutants

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Early detection of toxicity in air, water and soil is critical to prevent dramatic environmental contamination. Environmental biosensors allow us to assess such a toxicity. Among all these biosensors, recombinant bioluminescent bacteria focused the attention of environmental biotechnologists since these bio-systems have interesting characteristics such as specificity, robustness, autonomy, on-line measurability and high sensibility.

Arsenic and mercury constitute an important part of the natural and anthropogenic pollution and their detection is a concern of public health. Bacteria were modified to respond, by emitting luminescence in less than 15 min, to arsenic and mercury even at low concentrations (resp. <10 μg/l and <0.1 μg/l). Bacteria were also freeze-dried and continue to be active after 6 months of RT storage.

Activity from a slide supporting freeze-dried bacteria is detected by an original concept involving high sensitive detector. Thus, we present here an original combination of biological and biophysical technologies assuming the challenge of the “on-field measurements”. This technology can be applied to single measurements or continuous detection for different pollutants.

Keywords: bioluminescence, detection, heavy metals
Aggregation-based *in silico* study for better understanding of related membrane interfering analogous of Amphotericin B

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There is a general scarcity in effective and nontoxic antibiotics to treat systemic mycotic infections, especially in patients with immunological deficiencies. This is one of the common problems in antifungal chemotherapy. Systemic fungal infections are common side effects in anticancer chemotherapy treatments, which affect the immune system. Amphotericin B (AmB) which is one of the polyene macrolide antibiotics is the currently available and most effective drug for the treatment of a broad range of systemic antifungal infections in humans. One of the advantages of this drug is to prevent the antibiotic resistance in fungal strains. This useful property of AmB makes it more important in medical treatments. The mechanism of action and toxicity of AmB is not yet clearly understood, however it is believed to be through interaction with ergosterol in the cell membrane of fungi as compared to cholesterol in human cell membranes. The higher affinity towards ergosterol as well as aggregation of AmB in the membrane with each other to form a pore are the main information we have of the mechanism of action for this drug.

This study aimed at *in silico* identification of new analogues for AmB was carried out, and then practical studies on the activity of such molecules were performed. At first, the structure of AmB was considered as a template for a series of computational experiments to reach molecule which can form aggregations and also interact with membrane components. AmB was fragmented and according to binding energy obtained through *in silico* dock experiments the best molecules were identified. A similarity search was performed on the optimum molecules from previous step that showing the best binding profile; hence, several molecules could be selected and evaluated for their interaction with the sterols by UV spectroscopic study. Finally, an *in vitro* antifungal assay was performed on these molecules. The result of the evaluations was finding several compounds that their antifungal effect had not been reported before and proposed new mechanism of action possibly involve in binding membrane components such as ergosterol. In this paper, we applied a new strategy to find new analogues of a structurally complex molecule such as AmB. These molecules were able to behave in a similar manner and possibly the studied functions pattern is responsible for their efficiency. Among the found molecules were a well-known antibiotic, which proposes new protocols tracing the mechanism and for interpreting the observed activity, while allowing such molecules be handled better for possible interactions in clinical settings.

**Keywords** aggregation, self-dock, Amphotericin B, interaction energy, ergosterol, cholesterol

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Assessment of imazalil activity on the growth of *Penicillium expansum* and production of patulin in potato-glucose-agar medium

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The most important pathogens of apple are *Penicillium* spp., mainly *P. expansum* and *P. solitum*, which cause blue mold in apple. Effective fungicides are needed to control that apple disease. Imazalil is a systemic fungicide that is commonly used on several crops, mainly fruits such as apples, oranges, pears, etc. This fungicide is used to control fungal infections in post-harvest stages and it is indicated against *Penicillium*, *Phomopsis* and *Diplodochi fungii*. However, there is a lack of in-depth studies to evaluate the effectiveness of imazalil to avoid patulin (PAT) accumulation in fruits by *P. expansum* isolated from apples. *P. expansum* is known to be the main species capable of producing PAT as a secondary metabolite. Due to its acute and chronic toxicity, teratogenicity, immunotoxicity and mutagenicity PAT content in fruit juices, solid apple products and food for children has been limited by the European Commission.

The main objective of this work was to study the influence of imazalil and factors such as water activity (a), temperature and time on the growth and production of PAT by *P. expansum*. Potato-glucose-agar (PDA) medium was used in this study. Preliminary studies were performed at 0.99 aw and 24 °C using imazalil sulfate over a wide range of doses (1 – 500 ng imazalil sulfate/g medium) to control growth of a strain of *P. expansum* in the medium and its ability to produce PAT. As the lag phase increased considerably at doses > 100 ng/g medium, detailed studies were carried out in the 0.93 – 0.99 aw and 4 – 24 °C.

Statistical analysis (ANOVA) and response surface analysis of the data revealed that imazalil concentration and temperature factors had significant effects on lag phase duration in the PDA medium. The highest lag-times were observed at 4 °C, and at high levels of imazalil. Temperature and covariable lag phase had significant effects of the growth rate in PDA medium. The growth rate of *P. expansum* on potato-based culture increased at relatively high temperatures. PAT accumulation was studied as a function of the three previously considered factors and time. All factors, except temperature, had significant effects on PAT accumulation in PDA medium. On the basis of the results obtained, low doses of imazalil sulfate do increase the lag phase of *P. expansum* in PDA medium, do not substantially decrease fungal growth rate once growth is apparent and do not increase PAT accumulation in the potato-based medium.

**Keywords** imazalil; *Penicillium expansum*; patulin

**Acknowledgements:** the authors wish to thank financial support from FEDER and Spanish Government “Ministerio de Ciencia e Innovación” (Project AGL2007-66416-C05-01/ALL and two research grants).
Assessment of physiological heterogeneity of a population in multi-species microbial community by fluorescence techniques

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Individual microorganisms differ from each other in terms of physiological properties in population as well as multi-species community. The physiological differences of individual cells in a bacterial population are mainly due to progression through the cell cycle, differentiation of life cycle, and interaction of cell with inhomogeneous microenvironment. Therefore, the viable fraction of microbial community exploited in biotechnological process of wastewater treatment as well is heterogeneous in terms of physiological states. The determination of physiological states of individual cells of population within a microbial community may help to improve biotechnological process.

Fluorescence techniques are proper methods for determination of physiological states of individual microbial cells in a population.

Escherichia coli DSMZ 1329 and Pseudomonas veronii DSMZ 11331 were two strains used in this research. Plasmids of pEGPlacTet and pBA28 were electroporated into E. coli and P. veronii cells, respectively using standard molecular biology methods. LIVE/DEAD viability kit (SYTO 9/PI) was used for determination of membrane integrity (viability) of cells. Membrane potential and respiratory activity of cells were evaluated by bis-(1,3-dibutylbarbituric acid) trimethine oxonol (DiBAC4(3)), and 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) fluorescence probes, respectively. The cell viability in population introduced into microbial community was measured by combination of green fluorescence protein (GFP)-PI or GFP-CTC in GFP-labeled E. coli culture. GFP-tagged bacterial cells under all growth conditions produce fluorescence, which does not indicate physiological status of the cells. Therefore, fluorescence probes such as PI and CTC were used to determine the viability and respiratory activity of cells in population introduced into microbial community. The self-aggregated cells of P. veronii were grown in batch culture as well as in continuous culture in sequencing batch reactor (SBR), which was designed for formation of aerobic granules. The fluorescence based techniques such as flow cytometry (FCM) and confocal laser scanning microscope (CLSM) have been used in single-cell analysis.

The flow cytometry analysis of E. coli and GFP-tagged E. coli stained by fluorescent probes showed the existence of various subpopulations during different phases of growth and starvation. The fraction of CTC-reducing cells (CTC-cell subpopulation) was 95% and 83% in the exponential and stationary phases of batch culture, respectively. The fraction of depolarized cells, which was stained by DiBAC4(3), was 5% and 25% in the exponential and stationary phases, respectively. In the samples taken from exponential and stationary growth phases of batch culture, 14% and 50% of respiratory active (CTC-) cells were used for determination of respiratory activity by FCM. Two different cell subpopulations of “live” and “dead” cells were detected in both populations of E. coli and GFP-tagged E. coli in the stationary growth phase and in the phase of starvation. However, the subpopulations of “dead” and “live” cells in GFP-tagged E. coli population were more distinct in comparison to those in E. coli population stained by SYTO 9 and PI. The data for respiratory activity of cells in the GFP-tagged E. coli population stained by CTC were consistent with those from the E. coli population.

The spatial pattern of growth and respiratory activities within the cellular aggregate (granule) of P. veronii stained by CTC was analyzed by CLSM. It demonstrated the presence of three distinct layers: (1) the outer layer of cells with low respiratory and, probably, growth activities on the depth of 10 μm from the edge of the aggregate; (2) the intermediate layer of cells with the highest respiratory, and probably, growth activities between 10 to 20 μm from the edge of the cellular aggregate; (3) cells with the lowest respiratory and growth activities in the core of cellular aggregate. The capability of GFP gene as marker for tracking and visualizing cells introduced into community allows the GFP-tagged P. veronii to be specifically monitored during granules formation in SBR by CLSM. The single-cell approaches and techniques discussed in this paper for analyses of physiological heterogeneity of bacterial populations and aggregates used in environmental biotechnology could be helpful in optimization of the process.

Keywords: Single-cell analysis, physiological heterogeneity, flow cytometry, fluorescence probes, Escherichia coli, green fluorescence protein, aerobic granule, Pseudomonas veronii

Bacterial growth as a nonlocal coherent phenomenon

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On the basis of the first- [1] and second-order [2,3] Gompertzian kinetics it has been proved that the growth of the bacterial colony belongs to the class of quasi-quantum nonlocal coherent phenomena. The notion quasi-quantum refers to the possibility of application of the quantum language and formalism in description of macroscopic biological systems, e.g. organism, tumor, bacterial colony. The approach proposed reveals that the formation of the specific growth patterns during bacterial growth is a result of the nonlocal long-range cooperation between the microlevel - the individual cell and the macrolevel - the system of cells as a whole. Such nonlocal cooperative self-organization and intricate communication capabilities are observed during collective production of extracellular “wetting” fluid for movement on hard surfaces, long-range chemical signaling, e.g., quantum sensing, chemotactic signaling and collective activation and deactivation of genes. Those processes utilize the long-range communication and cooperation between microorganisms resulting in spatially coherent growth of the colony. An extension of the approach proposed to include von Bertalanffy model, which provides a much accurate bacterial growth curve than the Gompertz function is also presented.

Keywords: bacterial growth, pattern formation, Zwietering-Gompertz curve; quasi-quantum phenomena; coherence; nonlocality

Bacterial liquid-like envelopes detected by dynamic atomic force microscopy: false capsules/EPs.

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Motivation. Bacterial capsules are considered virulence factors, contributing to their attachment and evasion from host defenses and protecting them from desiccation. For example, pneumonia-causing bacteria Streptococcus pneumoniae has capsulated and capsulated strains. Those lacking a capsule are easily destroyed by the host and do not cause disease, whereas the capsulated ones resist phagocytosis.[1] Capsules have been found both in gram-positive and gram-negative bacteria, and even in some fungi such as Cryptococcus neoformans.[2] Composed of highly hydrated polysaccharides, they exhibit an extremely delicate physical nature, which makes them very sensitive to dehydration in SEM/TEM. In the case of very thin (nm-range) capsules, they might be elusive to staining methods and optical microscopy.

Antecedents. Atomic Force Microscopy (AFM) has proved to be capable of imaging the surface of liquids. In exploiting this capability, several works have reported successful observation of bacterial capsules/EPs in the form of tiny amounts of liquid-like extracellular substance, using ambient AFM.[3-6]

Objectives. To go deeper into this issue, three Staphylococcus epidermidis strains have been scrutinized for the possible presence of capsules using AFM. Some authors have suggested all staphylococci to be encapsulated.[7-8]

Conclusions achieved. Extensive imaging and X-ray Photoelectron Spectroscopy (XPS) chemical analysis have shown the liquid-like structure to be produced as result of an interaction of water with the ions present in the buffer used (Kpi). Such ultrasmall liquid volumes should however evaporate almost instantaneously under ambient conditions. We suggest Deliquesence as the basic chemical property responsible for the creation and stability of such ultrasmall liquid volumes surrounding the microorganisms. Deliquesence is a phenomenon by which certain substances absorb water from the environment and dissolve into them. This is in contrast with hygroscopicity, in which water is only adsorbed. For our buffer, one of its two components, K2HPO4, is a very deliquescent substance (unlike to other, KH2PO4, which is not). This also explains the results of others using solutions of CaCl2 (one of the most deliquescent salts known) or HEPES (with a highly deliquescent piperazine moiety). This non-biological origin explains the high similarity of our results with others using other (both gram-positive and gram-negative) bacteria and buffers, and the high similarity of our own results with very different strains.

Characterisation of initial degradation stage of Scots pine (Pinus sylvestris L.) sapwood after attack by brown-rot fungus Coniophora puteana

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Despite the long-term studies, the mechanisms of wood biodegradation by brown-rot fungi are yet not fully understood. The dynamics of biodegradation from early to late degradation stages as well as the critical stage in the degradation period, which causes irreversible consequences in the wood structure, are unclear. In our study, the investigation of early degradation period (10 days) of Scots pine (Pinus sylvestris L.) sapwood by the brown-rot fungus Coniophora puteana (Schum. fr.) Karst. (BAM Ebw 15) was based on detection of changes in the wood chemical composition, ultrastructure, and generation of reactive oxygen species (ROS).

Light microscopy observations confirmed extensive spreading of the fungal hyphae in wood tracheid lumina and rays already after ten exposure days. Scanning UV microspectrophotometry (UMSP) analyses of lignin distribution in wood cells revealed that the linkages of lignin and polysaccharides were disrupted already in the early period of fungal attack. Increase in lignin absorption A280 value from 0.24 (control) to 0.44 in decayed wood was attributed to its oxidative modification, which proceeded in the presence of ROS formed in Fenton reaction. The wood weight loss in the initial degradation period was 2%, while cellulose and lignin losses were 8.5% and 2.9% respectively. Lignin methoxil groups decreased from 15.1% (control) to 14.2% in decayed wood. Fourier Transform Infrared Spectroscopy (FTIR) analyses showed a moderate loss in the hemicelluloses content. Changes were observed in the band at 1730 cm-1 that is mainly due to the acetyl groups of glucomannan. Electron paramagnetic resonance (EPR) spectra confirmed the generation of ROS such as hydroxyl radicals HO in the very early wood degradation period. DMPO OH signal intensity was 10.1 relative units after 10 wood degradation days. Our results showed that irreversible changes in wood structure started immediately after wood colonisation by fungal hyphae. The understanding of biochemical mechanisms of wood biodegradation by brown-rot fungi is important in the development of novel wood protection methods.

Keywords brown-rot; Scots pine; early degradation; wood structure

References
Color measurements as a reliable method for estimating chlorophyll degradation to phaeopigments

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Chlorophyll degradation is usually used as a symptom of stress conditions due to environmental pollution. The ratio between absorbances at 435nm/415 nm is taken as an index of chlorophyll degradation: a decrease in this ratio indicates the degradation of chlorophyll to phaeopigments. We set up this study in order to investigate whether color measurements could be used as an indicator of environmental pollution instead of traditional methods of analysis which require sampling. CIELAB color parameters (L*, a*, b*, C* and h), the chlorophyll a content and the phaeophytination index (OD435 nm/OD 415 nm) of two biofilm-forming cyanobacteria on stone: *Nostoc* sp. PCC 9104 and *Nostoc* sp. PCC 9025, were analysed. Our results indicate that color measurement could replace the traditional methods (such as the determination of chlorophyll degradation) as an indicator of environmental pollution, since correlations between values of OD 435 nm / DO 415 nm and four of the five CIELAB color parameters are as close as correlations between the ratio DO 435 nm / DO 415 nm and chlorophyll a concentrations (Table 1).

**Keywords** chlorophyll, nondestructive methods, CIELAB color measurements, ratio of the phaeophytination index.

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Design of predictive models for deoxynivalenol accumulation in barley grain cultures of Fusarium culmorum under different conditions

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Deoxynivalenol (DON) is a trichothecone mycotoxin produced by various species of Fusarium such as F. culmorum and F. graminearum that contaminate cereal crops worldwide. DON induces inhibition of protein synthesis in cells and affects emetic response, growth, immune function and reproduction and limits for it have been set up by the European Commission in cereals, refined commodities and feedstuffs. Predictive models can be helpful to forecast the level that mycotoxins can reach in food and feed and beverages. A study was made to find out the possibility of using artificial neural networks encompassing both multilayer perceptrons (MLP-NN) and radial-basis function networks (RBFN) to predict the accumulation of DON in barley seeds contaminated with a strain of F. culmorum isolated from Spanish barley. The input (predictor) variables to the models were incubation temperature (20–28ºC), aw (0.94–0.98), size of the plug of used to inoculate seeds (7-15 mm diameter) and time; the output was the concentration of DON determined by gas chromatography. MLP-NNs with one and two hidden layers were trained using three different algorithms and two approaches within each algorithm (without and with hold-out validation). In any case, the input data in the subsets used for training and validation were chosen in a random way in every run. So it was the initialization of the weights. The general criterium adopted for design optimization was the value of the mean-square error (MSE) for the test subset but other error parameters such as the root mean square error, the standard error of prediction, as well as R² values were also computed.

For design of RBFN, which have only one hidden layer, treatment was simpler. Only the spread parameter was changed to find out the optimum value, which was the unity. Then, this value was applied for training networks. No validation procedure is needed and the performance for correct prediction was based on the lowest MSE for the test data set.

It was found that the training algorithm and the validation procedure influenced the performance of the predictive model. Within the MLP-NNs, the lowest MSE for test was accomplished by a single layer perceptron with 8 nodes in the hidden layer training without hold-out validation with the RBF algorithm. The more complex MLP having two hidden layers did not provided lower MSE values. For a given architecture and a given training algorithm the MSE values were higher when hold-out validation was accomplished. RBFN proved useful and reached lower MSE than MLP-NN to predict DON accumulation but a higher number of hidden nodes (85) was needed. The MSE decreased smoothly with the number of hidden nodes following a hyperbolic-like function.

From the study it was concluded that accurate prediction of DON accumulation in barley seeds by F. culmorum is possible using MLP-NN or RBF networks. The contribution here shown is part of a wider task to apply these models to mycotoxin forecasting in a variety of food commodities. Therefore, more research work on this topic is needed to encompass more fungal strains and other mycotoxins.

Keywords: predictive models; barley; Fusarium culmorum

Acknowledgements: the authors wish to thank financial support from FEDER and Spanish Government “Ministerio de Ciencia e Innovación” (Project AGL2007-64416-C05-01/ALI and two research grants).
Differences in stationary phase cells of *Saccharomyces cerevisiae* var. bayanus grown in aerobic and hypoxic bath cultures assessed by electric particle analysis, light diffraction and flow cytometry

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*Saccharomyces cerevisiae* is a yeast of widely recognized biotechnological interest and is also used as a model to understand the cell cycle progression of eukaryotic cells. In an asynchronously growing *S. cerevisiae* population, individual cells differ in their position (or phase) within the cell division cycle, their genealogical age and their size. All these variables drive the cell size distribution. Because of the tight coupling between cell growth and division, the study of cell size distributions of yeast populations at steady state, or under perturbed conditions, can reveal a wealth of information on the cell cycle regulatory mechanisms and adaptation to the environment.

From different principles, electric particle analysis and light diffraction are two current techniques that permit the obtaining of cell size distributions. In the field of microbiology, electric particle analysis is more usual, whereas light diffraction is used in most abiotic particles. Another current technique used to measure individual parameters, to identify subpopulations and to count microorganisms is flow cytometry. By this technique, the incident light scattered from a cell is collected in two different angles, in a narrow forward angle (forward scatter, FS) and in a proximally right angle (side scatter, SS) from the light beam. FS is a complex parameter as this function varies not only with cell size but also with cell shape, refractive index and number of intracellular dielectric interfaces. SS is also an intricate parameter. This signal is thought to indicate variations in cell surface or internal structure, usually referred to as "cellular granularity." In this contribution, we report cell population analyses of *S. cerevisiae* in stationary phase grown in aerobic and hypoxic bath cultures by three experimental techniques: electric particle analysis, laser diffraction and flow cytometry.

The medium used in aerobic conditions contains: 10 g l\textsuperscript{-1} glucose, 5 g l\textsuperscript{-1} yeast extract and 3 g l\textsuperscript{-1} casein peptone, and pH was initially adjusted to 3.5 with orthophosphoric acid. To ensure hypoxic condition the medium was supplemented with 0.5 g l\textsuperscript{-1} sodium thioglycollate and 0.001 g l\textsuperscript{-1} resazurine. Both media were autoclaved for 15 min at 121°C. The inocula, prepared and cultured in the same medium growing in aerobic and hypoxic conditions, respectively, were inoculated in 1000 ml flasks with 500 ml of the fresh medium and incubated at 27°C, using magnetic shaking (150 r.p.m) for approximately 60 hours. Along this time, and after the steady state was confirmed, samples were removed to be analysed using an electronic particle analyser, a laser diffraction particle size analyzer and a flow cytometer.

Multiziser data shows that the cells under hypoxic conditions are greater (means from 4.93 to 5.23 μm) with size distributions moving to the left along the stationary phase, whereas cells grown aerobically are smaller (means from 4.46 to 4.67 μm) with more stable size distributions. Although cell size distributions obtained by light diffraction show a different shape (slightly positively skewed with no left tail), the use of laser diffraction confirms that the cell sizes in aerobic conditions are smaller (means from 4.50 to 4.66 μm) than cells grown in hypoxic medium (means from 4.98 to 5.00 μm). However, with this method we observe that the stationary phase is not stable in aerobically grown cells, and there is a greater variability in cell size distributions from the twenty-hour sample to sixty-hour sample. Basic shape of FS distributions shows a similarly basic shape in both growth conditions, centered in a range of relative intensities between 3500-3700 with a long right tail. In addition, cells grown in hypoxic conditions show a subset with relative frequencies between 4500 and 6000, whereas in aerobic conditions there is a higher proportion of cells with relative intensities between 6000 and 10000. This is consistent with the existence of two subpopulations, not observed in previous techniques. The two subpopulations show more differences in aerobic conditions than in hypoxia. Moreover, the subpopulations in aerobic conditions join at the advanced stationary phase. Similarly, SS distributions in both experiments are essentially the same: a triangular distribution slightly positively skewed and centered at relative intensities between 4000-4200. However, in this measure one can also observe a subpopulation of yeast cells with relative intensities between 6000 and 10000 in aerobic conditions but not in hypoxic conditions. Therefore, it is shown that in the stationary phase of culture in hypoxic conditions the population is more homogeneous than under the aerobic cultivation.

Keywords: Yeast populations; Cell size distribution; Electric particle; Light diffraction; Flow cytometry analysis; Stationary phase

Dissecting Gene Expression in Micro-Colonies of *Aspergillus niger*

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The filamentous fungus *Aspergillus niger* forms centimeter scale macro-colonies on solid media, whereas (sub)millimeter micro-colonies are formed within a shaken culture. These colonies secrete large amounts of proteins to degrade polymers in the medium into compounds that can be taken up to serve as nutrients. Previously, it has been shown that macro-colonies of *A. niger* that had been grown on solid medium are heterogenic with respect to gene expression and protein secretion. Here, we assessed whether heterogeneity can also be found between and within micro-colonies of a liquid shaken culture of *A. niger*.

Micro-colonies of *A. niger* strains expressing GFP from the promoter of the glucoamylase gene *glaA* and the ferulic acid esterase gene were sorted on basis of their diameter and fluorescence of the reporter protein using the Complex Object Parametric Analyzer and Sorter (COPAS). Fluorescence intensity of the reporter correlated with the volume of the micro-colony only. In contrast, the lowly fluorescent micro-colonies of the strain expressing *glaA* promoter comprised about 79 % of the culture. This indicates that heterogeneity in this strain depends on the volume of the micro-colony only. Therefore, it is shown that in the stationary phase of culture in hypoxic conditions the population is more homogeneous than under the aerobic cultivation.

Keywords: Heterogeneity, *Aspergillus niger*, COPAS, LMPC, towards single cell analysis

This research was supported by the IOP Genomics program of the Dutch Ministry of Economics Affairs and by the Dutch Technology Foundation STW, Applied Science division of NWO and the Technology Program of the Ministry of Economics Affairs.
DTAF: An Efficient Probe to Study Cyanobacterial-Plant Interaction Using Confocal Laser Scanning Microscopy (CLSM)

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Different microscopic techniques have been utilized to study cyanobacterial associations with plant roots but confocal laser scanning microscopy (CLSM) is the least used due to unavailability of suitable fluorescent dye. Commonly used lectins have problems with their binding ability with root cells and their visualizations with CLSM. DTAF (5-(4,6-dichlorotriazinyl) aminofluorescein) is a fluorescent dye that has been widely used in staining various biological samples for fluorescent microscopy. It reacts with polysaccharides and peptides at ordinary conditions. The possible application and efficiency of DTAF for CLSM studies was examined in various aspects of cyanobacterial-plant interactions. Seedling of Pisum sativum, Vigna radiata, and Triticum aestivum were co-cultivated and stained with DTAF as fluorochrome. Extracellular and intracellular interactions of cyanobacteria and plant roots surface was observed by CLSM. Results were compared with staining by other commonly used lectins. DTAF can be used with all plants giving fluorescent signals at 517 nm that is highly adventitious as cyanobacteria has an auto-fluorescence at 665 nm and both can be simultaneously used in CLSM by visualizing in different channels. It worked efficiently with all three plants used and with filamentous and unicellular cyanobacterial strains. Cyanobacterial presence was not only clearly observed on root surface but also inside the root tissue and epidermal cells. Easy protocol and absence of tissue processing make DTAF a useful probe for studies of cyanobacterial associations with plant roots by CLSM.

Key words: CLSM; Cyanobacteria; cyanobacteria-plant associations; DTAF; 5-(4,6-dichlorotriazinyl) aminofluorescein; fluorescent dye

Ethanol biosensor based on rhodium dioxide and alcohol dehydrogenase

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During the last two decades, one can observe an increasing interest in applications of disposable biosensors, particularly those based on screen-printed carbon electrodes (SPCEs) [1]. In electrochemical biosensors, advantages of the enzyme specificity for recognition of particular target molecules are combined with the direct transduction of the reaction rate into a current [2]. Resulting biosensors are effective and selective for the substrate detection [3–4]. With the biosensors preparation, an enzyme immobilization belongs to the most important operations. An inconvenient manner of the enzyme entrapment can cause its denaturing, indirect inactivation, or its washing out from an electrode body. The choice of the enzyme immobilization method depends on the enzyme characteristics, on the type of transducer, and on the conditions at which the biosensors work, and finally on the physical characteristics of the analyte.

In this contribution, SPCEs containing RhO2 as the modifier [5,6] were used. Different methods of immobilization were tested, including entrapment in Nafion, cross-linking with glutaraldehyde, immobilization with cellulose acetate, and electropolymerization using pyrrol or m-phenylenediamine. Finally, the ethanol biosensor was prepared using alcohol dehydrogenase (together with cofactor NAD+ and trehalose). Concerning its quality in terms of retaining the enzyme activity, response time, sensitivity and dynamic range of concentrations, the immobilization of enzyme via electropolymerization using m-phenylenediamine seemed the best. Resulting biosensors have been tested in model samples of ethanol. In such applications, biosensors showed a linear response in the range of 15 – 120 g L−1 ethanol with a detection limit of 3.3 g L−1 (evaluated as 3σ/3σ) and a response time of 19 s.

Acknowledgement
This work was supported from the Ministry of Education, Youth and Sport of the Czech Republic (projects MSM0021627502 and LC06035) and the Czech Science Foundation (project 203/08/1536).

Keywords alcohol dehydrogenase, amperometric biosensor, screen-printed carbon electrode, rhodium dioxide, ethanol

References
Flow cytometry for analysis and sorting of large particles, sized from 20-1,500 microns (e.g. Aspergillus, Daphnia, Aquatic Larvae, Pollen).

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Introduction:
Many objects are too large and too fragile for conventional flow cytometry. Manual microscopic manipulation of large objects is tedious, subjective, and limits the size and scope of experiments. Likewise, conventional flow cytometers are limited by the size of the objects that can be analyzed, thereby limiting their use in several applications.

Instrumentation
Instruments (COPAS™) are now available to automate the analysis and sorting of large (20-1,500 micron) particles in a continuously flowing stream at a rate of 10-100 objects/second. Using object size, optical density, and intensity of fluorescent markers as sorting criteria, selected objects in this size range, can be dispensed in multi-well plates for further analysis. A gentle pneumatic sorting mechanism located after the flow cell avoids harming or changing sensitive objects, thereby making the instrument suitable for live biological materials or sensitive chemistries. Multiple fluorescence excitation and emission wavelengths are available. Today, automation, increased sensitivity, and quantitative measurements enable larger / faster screens of many large particle applications. Applications include: Zooplankton determination, Daphnia toxicity test, Marine larve counting and dispensing, Pollen analysis and sorting for Radiocarbon dating, Aspargillus analysis and several large cell (cluster) applications. The COPAS instruments allow using three PMTs at the same time. In addition, Profiles can be generated for each individual object, showing the localization of fluorescence in the object.

Keywords Large Particle Flow Cytometry, Analysis and Sorting, Fluorescence

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Generation of computational metabolic models of three strains of Escherichia coli and growth comparisons in the presence or absence of oxygen

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Within the genus Escherichia, only one strain, E. coli K-12 MG1655, has had a genome-scale metabolic computational model constructed. While this single model has proven useful for guiding the bioengineering of strains for increased production of desired end-products, we sought to explore how constructing additional metabolic models of E. coli could potentially enhance these efforts. In this work, the metabolic model for E. coli K-12 MG1655 was updated to account for 1,322 ORFs, 91% of which are based on experimentally validated function, and includes a new pathway for phenylacetate metabolism. New genome-scale metabolic models were constructed for two pathogens, enterohemorrhagic E. coli O157:H7 strain EDL933 (1,328 ORFs) and the uropathogenic E. coli strain CFT073 (1,264 ORFs). When compared to the E. coli K-12 model, the metabolic networks for E. coli O157:H7 EDL933 and E. coli CFT073 contained 57 and 29 lineage-specific ORFs, respectively. All three E. coli models were used to simulate growth in different conditions and the results were compared to experimental data for utilization of 76 carbon sources in conditions with or without oxygen, revealing different metabolic capabilities for each strain. Both in silico predictions and experimental results for growth during batch fermentations in minimal media with 0.2 % (w/v) glucose reveal that both pathogens attain maximum biomass (g/L) faster and in greater amounts than E. coli K12 MG1655. Our findings suggest that quantitative models of different strains of E. coli can accurately predict strain-specific phenotypes and offers bioengineers a larger suite of metabolic capabilities with which to work with for designing new industrial E. coli strains.

Keywords Large Particle Flow Cytometry, Analysis and Sorting, Fluorescence
Location sites of nucleic acid intercalators in yeast cells: computer-aided fluorescence microscopy study

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The aim of this study was to test if the live yeast cells of Saccharomyces cerevisiae can be used as a model for locating intracellular sites/targets of the nucleic acid intercalators. To this end, intracellular distributions of the anticancer drug doxorubicin (DR) along with 4',6-diamidino-2-phenylindole (DAPI), and ethidium bromide (E) were investigated using fluorescence microscopy combined with computer image analysis (ImageJ software, NIH, USA).

Yeast cell culture was found to be heterogeneous in stainability by DAPI, DR and E. Presence of the drugs in some cells could be revealed only after special processing of the images by increasing brightness/contrast by ImageJ. Most likely, this stainability pattern was a consequence of the difference in the cell wall/plasma membrane permeability for the drugs. Drug export permease Sge1 could play a role in reducing permeation of the drugs.

Upon permeation of DAPI into the cells, structures having two fluorescence colors, blue and yellow, could be visible. The increased blue color fluorescence of DAPI is known to be due to the intercalation into核酸 acids. Yellow fluorescence of DAPI was shown to be caused by the interaction with polyphosphates. Three sites of DAPI location could be revealed in the live yeast cells: DNA in the nuclei and in the mitochondria, that fluoresce blue, and the polyphosphate complexes (if present), that fluoresce yellow. Polyphosphate complexes are known to be located in vacuoles.

Two sites of DR location were found in the live yeast cells: the nuclei and the area corresponding to mitochondria. In contrast to DAPI which stained mitochondria as clearly separated “units”, DR fluorescence in this area, in most cells, was even or diffuse. However, in some cells, when permeation of the drug seemed to be restricted, the pattern of the mitochondria staining was similar to that of DAPI. Using pseudospectral analysis with the RGB-Split option of ImageJ, it was shown that the DR fluorescence spectrum in the nuclei was shifted to the red as compared to the fluorescence in the area of mitochondria. It was suggested that, along with nucleic acids, DR had affinity and was bond to some other macromolecules or macromolecular complexes that resulted in its fluorescence quantum yield increase with changes of the spectral features.

There were three visible potential sites of E location in the live yeast cells: DNA in the nuclei and the mitochondria, and some diffuse matter. The latter had higher fluorescence intensity in the cells that seemed to be “highly permeable” for the dye.

It was revealed by ImageJ analysis that spectral features of E in the nuclear and in the mitochondrial regions were the same. In the binding sites of the “diffuse matter” of the “highly permeable cells”, a small “blue shift” of E fluorescence spectrum was detected.

Upon addition of DR or E along with or before DAPI, the red component of their fluorescence in the nuclei was less as compared to that if DR or E were added alone. Taking into account that DAPI fluorescence in the red region is close to zero, this could be interpreted as competition of the drugs for the same sites of interaction/intercalation in nucleic acids. At the same conditions, there was not significant decrease of DAPI fluorescence in blue region. This is an indication of no fluorescence resonance energy transfer (FRET) from DAPI to DR or E. The FRET might have been expected in the case that donor (DAPI) and acceptor (DR/E) molecules were in close proximity (less than 10nm). Therefore, location sites of DAPI and DR/E in nucleic acids in these experimental conditions are at distances higher than 10 nm.

CONCLUSIONS

1. The live yeast cells of S. cerevisiae can be used as a model for locating intracellular sites/targets of the fluorescing nucleic acid intercalators by fluorescence microscopy combined with computer image analysis.

2. In live yeast cells, the intercalators may interact with the nucleic acids in the nuclei and in the mitochondria. Also, there are some other macromolecules and/or macromolecular complexes which can bind the drugs, too. For DAPI, this complex is known to contain polyphosphates. The chemical composition of sites of DR and EB binding other than nucleic acids is not known as yet.

3. Both DR and EB compete with DAPI for the same binding sites in the nucleic acids of the cells. One implication of this finding could be the development of new approaches for investigating intracellular location of nonfluorescing drugs targeting nucleic acids in combination with fluorescing ones.

Keywords: fluorescence microscopy; image analysis; yeast; Saccharomyces cerevisiae; anticancer drug; intercalator; doxorubicin; DAPI; ethidium bromide; intracellular location
Metabolic reconstruction of *Synechococcus elongatus*, towards a minimal photoautotrophic cell

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The cyanobacterium *Synechococcus* sp. strain PCC 7942 (previously known as *Anacystis nidulans* R2) is an unicellular, obligate photoautotroph requiring only inorganic nutrients and light for growth. It represents a model organism for studies of photosynthesis, prokaryotic circadian rhythms, cell division, carbon-concentrating mechanisms, and adaptive responses to a variety of stresses. So, and once its genome has completely been sequenced, a grand challenge related to this cyanobacterium is the computational prediction of highly complex cellular processes, from that available genomic and molecular information. Towards the end above mentioned, we manually checked the available genomic annotation of the strain and set-up, a preliminary *Synechococcus* PCC 7942-specific PathoLogic database containing the predicted metabolic pathways. This initial metabolic network is being refined relying on the biochemical literature directly from PubMed, and from information derived from the Kyoto Encyclopedia of Genes and Genomes (KEGG), the Enzyme Database (BRENDA) and Transport Classification System (TCDB). The connections between a particular enzymatic activity and a gene were more reliable if that association was based on the experimental data on PCC 7942, that is, if we could find a journal article testing it. Simultaneously, we are testing for stoichiometric coherence of the reconstructed metabolic network using Flux Balance Analysis (FBA) enabled with ToBiN (Toolbox for Biochemical Networks). Once the genome-scale constraint-based metabolic model is finished, we will use ToBiN software to analyze key features of the metabolism such as growth yield, resource distribution, network robustness, gene essentiality, and to predict a minimal photoautotrophic cell.

**Keywords** Cyanobacterium; Flux Balance Analysis; Metabolic reconstruction

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**Noncyanobacteria induced by ethidium bromide in yeast plasma membrane**

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Cell membranes have the ability to bend and curve, thus providing cleft-like-coated pits and plasmalemma caveolae, and facilitating many cell functions such as receptor-mediated endocytosis. On the other hand all intracellular membranes are also highly deformable, producing cargo vesicles destined to organelles and plasma membrane. Generation of membrane curvature is currently believed to involve the penetration of amphipathic helix into the cytosolic face of the membrane bilayer, producing an asymmetry between the two leaflets of membrane and generating bending and curvature towards the cytosol. This research studies the morphological effects of ethidium bromide on the yeast *Candida utilis*. It showed that ethidium bromide induced bending and curvature at the yeast plasma membrane.

*Yeasts* *Candida utilis* were grown in synthetic medium with 100 µg copper per liter and 1.5% ethanol as substrate. The effects of ethidium bromide were studied by growing *C. utilis* yeast cells in the same medium supplemented with various concentrations of ethidium bromide. After 16 h of culture the cells were harvested, washed twice in distilled water, and treated for thin section and freeze-fracture studies. Thin section: Pellets of yeast cells were fixed in 5% potassium permanganate at room temperature (~ 20-25°C). The cells were then centrifuged and washed twice in double distilled water. The pellet was cut in 5-6 small portions (2-3 mm). They were dehydrated in graded concentrations of ethanol and embedded in Epon according to published procedure. Thin sections were cut with a Porter-Blum ultramicrotome equipped with a diamond knife and stained with lead citrate. Freeze-fracture: The yeast cells were freeze-fractured either without cryoprotectant or after incubation in 30% glycerol for 5-30 min.. A suspension of yeast cells (approximately 3 µl) was transferred to a sides loaded gold apposed specimen holder and rapidly quenched in liquid Freon 22 (chlorodifluoromethane) cooled to its freezing point by liquid nitrogen. Freeze-fracture was done by standard techniques. Replicas were cleaned in Chlorox (sodium hypochlorite), washed in distilled water and mounted on 400-mesh grids. The freeze-fracture specimens and thin sections were examined in a Philips EM 300 electron microscope, calibrated by a carbon grating replica, at an accelerating voltage of 80 kV with a 30-µm objective aperture. Thin section studies showed the formation of negative curvature in ethidium bromide-treated cells, with depressions that were 5-110 nm-deep. Freeze-fracture studies showed both protoplastic face (PF) and exoplasmic face (EF) of plasma membrane. Intramembrane particles were abundant at the PF surface but their numbers were comparatively reduced at the EF surface. The surface of untreated yeast cells exhibited numerous linear depressions at the PF face, which were suurrelated at the EF face. These depressions or surrelations corresponded to grooves that were 220 to 450 nm-long and 70 nm-wide. The plasma membrane of ethidium bromide-treated cells showed on the one hand a number of grooves whose morphology were similar to the control, on the other hand numerous depressions at the PF face, some circular, but most polymorph. The dimensions and morphology of these depressions (negative curvature), termed “nanocups”, were better illustrated at the EF surface than at the PF surface. The nanocups were always associated with a groove and were usually circular, although sometimes oval, with diameters of 200 to 300 nm. Occasionally two or three nanocups with associated groove fused together and formed larger, usually polymorph structures whose dimensions were between 500 and 600 nm.

Our studies showed that small molecules such as ethidium bromide can provide insight into bending and nanocurvatures of the yeast cell *C. utilis* plasma membrane. In erythrocytes there is a large group of compounds that cause plasma membrane deformations. Some of these compounds such as uncoupler, barbital, bilirubin and salicylate produced crenation of the red cell membrane, while other compounds such as chlorpromazine, colchicin and vinblastine were cup-formers or produced invaginations. Cup formers were all amphipathic cations. Ethidium bromide is an amphipathic molecule that upon its interaction with groove domain produced negative deformation of the yeast plasma membrane. Deformation of yeast *C. utilis* plasma membrane was also observed with another drug, doxorubicin. Our observations taken together with those of other authors allowed us to stipulate that, besides mechanical deformation, there are two other mechanisms producing alterations of the plasma membrane, one chemical and the other biological. Finally, even though membrane curvatures were abundantly visible, the generation of vesicles and tubules was not observed, thus suggesting that the ethidium bromide-induced plasma membrane deformations were not involved in intracellular trafficking.

**Keywords** yeast; bending and curvature; ethidium bromide; plasma membrane; thin section; freeze-fracture; nanocups
Natural product mining by nanoliter-scale cultivation of single actinomycetes spores in a microfluidic system

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During last decades the pharmaceutical industry faced serious problems developing new antibiotics. Mainly target-oriented screening strategies were applied, which did not provide the expected outcome. Moreover, the hardly predictable possibility of quickly developing resistances against newly introduced antimicrobial drugs raised doubts about their profitability. Since the need for new antibiotic substances or even substance classes is undeniable, new strategies for efficient hit generation are required. Microfluidic platforms allow high-throughput screening with whole-cell-assays – a promising approach postulated by leading experts.

We present our current progress in establishing a pressure-driven microfluidic system which provides the required unit operations for systematic discovery of biologically active compounds from soil organisms in a closed assay according to the following scheme (Fig. 1): Initially, growth medium compartments containing one spore each statistically are generated within the carrier fluid tetradecane (1) by microinjection (2). In a subsequent incubation loop (3), the organisms are given several days to germinate and eventually produce antimicrobial substances. The presence of antibiotics in the compartments is checked by addition of a fluorescing reporter strain (4) followed by a detection and sorting step (5). Positive compartments are visually detected and are sorted out (6) for large-scale cultivation and strain analyses.

![Fig. 1: Overview of microfluidic assay for discovery of novel antibiotics from actinomycetes](image)

As a preliminary result, we show that compartmented nanoliter-scale cultivation of actinomycetes within 72 hours is feasible – even though a prototype glass chip with poor oxygen transfer properties was used. Microfluidic chips which are currently in development will be manufactured from polymeric materials with improved oxygen transfer properties. Further steps to establish a robust assay are pointed out.

References


Keywords: Microfluidic system, antibiotics, actinomycetes, secondary metabolites, natural products

Neutron activation analysis for applied microbiology

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1Joint Institute for Nuclear Research, Dubna, RF, in microbiology jointly with the scientists from Georgia are presented. They include medical biotechnology, and environmental biotechnology. In the biomedical experiments a blue-green alga *Spirulina platensis* biomass has been used as a matrix for the development of pharmaceutical substances containing such vitally important trace elements as selenium, chromium and iodine. The feasibility of target-oriented introduction of these elements into *Spirulina platensis* biocomplexes retaining its protein composition and natural beneficial properties has been proved. The adsorption of such toxic metal as mercury by *Spirulina platensis* biomass in dynamics of growth has been studied also. INAA has been successfully applied to investigate the biotechnology of toxic Cr(VI) transformation into less toxic Cr(III) complexes by Cr(VI)-reducer bacteria isolated from polluted basalts in Georgia. This method was used to track accumulation of chromium in the bacterial cells. To monitor and identify Cr(III) complexes in these bacteria, electron spin resonance(ESR) spectrometry was employed. For the first time, the elemental composition of Cr(VI)-reducer bacteria has been studied using epithermal NAA.

Keywords: *Spirulina platensis*, neutron activation analysis, microelements, Cr(VI), Cr(III)
Quantifying the heterogeneous response of E. coli at temperatures close to the maximum growth temperature

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Introduction and objectives

Close to the maximum growth temperature, growth of E. coli K12 is disturbed (Van Derlinden et al. 2008). The irregular curves at 45 and 46°C were explained by the co-existence of two subpopulations: a resistant population and a sensitive population. In Van Derlinden et al. (2009), this hypothesis was analyzed via a heterogeneous model which includes (i) a growth model for the resistant population, and (ii) a combination of a growth and an inactivation model for the sensitive subpopulation.

In this work, the behavior of E. coli at temperatures between 45 and 46°C is studied in more detail. Focus is on the characterization of the subpopulations, i.e., (i) how the ratio constant or dependent on temperature, and (ii) how do the subpopulations’ dynamics change as a function of time and temperature. The behavior of the two subpopulations is analyzed via the subpopulation type model.

Results

At all temperatures between 45 and 46°C, the total population follows a sequence of growth, inactivation and re-growth, which can be explained by the two subpopulations (for illustration, see Figure 1). The first growth phase and inactivation phase reflect the presence of the sensitive subpopulation (ns). Hereafter, the population's dynamics is dominated by the growth of the resistant subpopulation (nr).

Conclusion

Prior experiments revealed that at 45°C, a sensitive and a resistant subpopulation exist. In this study, additional experiments are performed. Via description with a subpopulation type model, it was identified that the initial ratio of resistant cells in the overall population changes with temperature in the studied temperature range (i.e., 45-46°C).

Keywords: Predictive microbiology; Risk assessment

References

Sensitivity analysis of Campylobacter spp. in poultry based meat preparations

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Introduction

In quantitative microbiological risk assessment, the risk of illness associated with a certain pathogen/food type combination is estimated using predictive growth and inactivation models, models for cross-contamination, dose-response models, etc. Parameter values (or preferentially their distributions) that represent the real food chain as accurately as possible are estimated to arrive at a realistic outcome. Not only the risk obtained as such from the risk assessment is useful, the created model also offers a way to infer which parameters have the greatest influence on the final risk, and hence, which strategies might be best to reduce the risk for consumers. Sensitivity analysis methods (see, e.g., Frey & Patil (2002) for a review) offer an estimation of the sensitivity of the output of a model (here, the risk of illness) in relation to the input parameters.

Material and methods

In this research, it is investigated which parameters are of major importance in risk of illness caused by Campylobacter spp. in poultry based meat preparations. To this purpose, sensitivity analysis is applied to the risk assessment published by Uyttendaele et al. (2006) with some minor modifications. Using maximum likelihood estimation for censored data (Buschert et al., 2009), a distribution is estimated for the initial concentration of Campylobacter spp. This concentration is applied as the rate parameter of a Poisson distribution to estimate the number of cells in a single serving of 100 g. Because of the generally low concentrations, the majority of initial contaminations will contain zero cells per serving, and pose as a consequence zero risk. Therefore, the summed risk of 5 x 10^3 servings given a set of parameters is considered as well. Due to the characteristics of Campylobacter no growth is considered, and it is assumed that cooking the food product properly eliminates all cells; however, it is included in the model that undercooking might occur in a number of cases, as well as cross-contamination from the raw product to the cooked product. Because of the considerable uncertainty about the dose-response model, three different models are applied in order to assess the influence of this uncertainty on the outcome of the risk assessment. A Monte Carlo simulation with 5 x 10^3 iterations is implemented. For a preliminary exploration, scatter plots, Pearson correlation coefficients and Spearman rank order correlations are investigated. Analysis of variance and Fourier Amplitude Sensitivity Test (FAST) is subsequently applied to rank the importance of parameters.

Results

Application of different methods of sensitivity analysis to this model consistently indicate that the final risk is determined in the first place by the initial concentration of Campylobacter spp. in poultry meat. The obtained risk also depends to a great extent on the choice of the dose-response model; hence, further research to decrease uncertainty about the dose-response model would improve estimates of risk. The prevalence of undercooking has a significant influence on the final risk; however, it is merely a minor influence compared to the latter parameters. Moreover, the influence of prevalence of cross-contamination is not significant. This indicates that – for this particular case study – increasing the awareness of consumers of the risks posed by undercooking and cross-contamination would only have a limited effect on reducing listeriosis cases if compared to the initial contamination.

Acknowledgements

This research is supported in part by KULeuven-BOF Projects OT/09/25 and EF/05/006 OPTEC Optimization in Engineering, by the Belgian Program on Interuniversity Poles of Attraction, initiated by the Belgian Federal Science Policy Office, and by the Fund for Scientific Research – Flanders (FWO-Vlaanderen, project G.0424.09N).

Keywords quantitative microbiological risk assessment, sensitivity analysis, Campylobacter, poultry

Selectivity-refined in silico analogue finding method for new antifungal molecules based on amphotericin B molecular features

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The action of immunosuppressant drugs, cancer chemotherapy agents and medical conditions such as AIDS can cause the immune reduction that led to an increased prevalence of opportunistic infections such as fungal infections. There are two kinds of fungal infections, systemic and superficial. Many drugs have been approved as systemic antifungal. A major group of these drugs are polyene antibiotics. Polyene antibiotics are produced by several different species of Streptomyces which have lactone ring (20-24 carbons). The ring contains a series of conjugated double bonds (n=3-8) which often have a glycoside residue. The polyene antibiotics connect to the cell membrane and interfere with the permeability and transport functions. These antibiotics have the advantage of making low drug resistance in fungal strains. Amphotericin B, one of the polyene antibiotics that are produced by Streptomyces nodosus, is a potent antifungal agent which has been used for over thirty years to treat serious systemic fungal infections by binding to sterols such as ergosterol in fungal cells forms a pore in the membrane and create a transmembrane ion-channel. Since all eukaryotic cells contain sterols, using amphotericin B can cause toxicity in mammalian cells, that is the most serious unwanted side effect of this drug.

In this study, we have attempted to find new antifungal compounds based on the structure and function of amphotericin B, using in silico methods. First of all, amphotericin B was set as a query molecule for the similarity search that was performed in Enhanced NCI database, Pubchem, ChemIDplus and ChemBank-Welcome by Tanimoto Index between 70-99 %. The small molecules having less complexity than amphotericin B were selected (about 20,000 molecules). Quantitative structure-activity relationship (QSAR) studies, search connections between molecular structure and physicochemical properties of compounds resulted in polyene antibiotics which form pores in the membrane. About 60 polyene molecules were found from literatures and their physicochemical properties such as (logP, H-bond acceptor, H-bond donor, rotatable bond count, topological polar surface area, etc) were stored as a datafile. The mentioned molecules helped us to form a scoring system to screen selected compounds extracted from similarity search technique. Docking of the selected compounds, as ligands, were performed with ergosterol and cholesterol, as receptors, using Hex (version 5.1.2008). Default parameters were selected to obtain the best binding energy. A datafile of the dock obtained results and physicochemical properties of selected compounds were prepared. Using this, we selected compounds which have: 1) the best interaction energy with ergosterol in comparison to cholesterol, and 2) the physicochemical properties and molecular descriptors similar to polyene antibiotics. A target list was narrowed down. Ultimately antifungal activity of the intended molecules was examined on Candida albicans, Saccharomyces cerevisiae, and Aspergillus niger. Several compounds demonstrated to have good antifungal activity. We applied the new method to find new bioactive compounds that have antifungal effect on membrane.

Keywords amphotericin B, similarity search, physicochemical property, interaction energy

Application of different methods of sensitivity analysis to this model consistently indicate that the final risk is determined in the first place by the initial concentration of Campylobacter spp. in poultry meat. The obtained risk also depends to a great extent on the choice of the dose-response model; hence, further research to decrease uncertainty about the dose-response model would improve estimates of risk. The prevalence of undercooking has a significant influence on the final risk; however, it is merely a minor influence compared to the latter parameters. Moreover, the influence of prevalence of cross-contamination is not significant. This indicates that – for this particular case study – increasing the awareness of consumers of the risks posed by undercooking and cross-contamination would only have a limited effect on reducing listeriosis cases if compared to the initial contamination.
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Single Live-Bacterial Cell Assay of Promoter Activity and Regulation: Escherichia coli gcl promoter

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In the early stage of molecular biology, bacteria such as the Gram-negative Escherichia coli and the Gram-positive Bacillus subtilis have been used as model organisms relying on the belief that bacterial cells are homogenous in populations. Recently, however, we realized that bacterial population is heterogeneous, each cell playing a different role for population survival. Previously we indicated that laboratory cultures of E. coli contain various cell types, each differing in cell shape, physical properties such as buoyant density, and molecular composition (1,2). Such variation in cell populations may be arisen from the fluctuation in the cell age, during transition from exponential growth to stationary phase, or from the differentiation into various cell fates under stressful conditions. These findings altogether raised a criticism over the established concept of the regulation of E. coli gene expression because the data of experiments so far performed represent the average of a number of cells with different patterns of the genome expression. Our effort has since been focused on the development of experimental systems to analyze the promoter activity and the genome regulation within a single bacterial cell.

For solving these questions, we constructed various types of the cell chip for immobilization of portions of cell culture. We have analyzed the strength and regulation of the promoter of E. coli gcl gene encoding glyoxylate carboligase using developed the cell chip. The gcl gene is organized as the first gene in a large operon for reutilization of allantoin, an intermediate metabolite of purine degradation, and is under the control of transcription factor AllR. Previously we identified that antagonistic effectors, allantoin and glyoxylate, are involved in regulation of the gcl promoter (3). Thus this is a good model system to examine the single live-bacterial cell assay to measure the promoter activity and regulation. For this purpose, we have constructed a two-fluorescent reporter plasmid for assay of the gcl promoter strength (4). After immobilization of portion of E. coli culture within the newly constructed cell chips, we followed the GFP intensity in the presence and absence of glyoxylate for prolonged time period. In parallel, we also analyzed the promoter regulation in random cultures using FACS flow cytometry, and measured the fluctuation level of promoter strength within a single population in random culture. Here, we report the real-time single-cell assay and the fluctuation level of the promoter strength among a cell population within a single and same culture.

Keywords: single cell; cell chip; promoter assay; gcl promoter; Escherichia coli

The MiST2 database: a genomics resource on microbial signal transduction

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Signal transduction systems regulate the majority of cellular activities including the metabolism, development, host-recognition, biofilm production, and so on. Thus, knowledge of the proteins and interactions that comprise these communication networks is an essential component to furthering biological discovery. The MiST2 database identifies and catalogs the repertoire of signal transduction proteins in microbial genomes. These are identified by searching protein sequences for specific domain profiles that implicate a protein in signal transduction. Compared to the previous version of the database, MiST2 contains a host of new features and improvements including the following: draft genomes; extracytoplasmic function (ECF) sigma factor protein identification; enhanced classification of signaling proteins; novel, high-quality domain models for identifying histidine kinases and response regulators; neighboring two-component genes; gene cart; better search capabilities; enhanced taxonomy browser; advanced genome browser; and a modern, biologist-friendly web interface. MiST2 currently contains 966 complete and 69 draft bacterial and archaeal genomes, which collectively contain nearly 230,000 signal transduction proteins. The majority (65%) of these are one-component systems, followed by two-component proteins (25%), chemotaxis (6%), and finally ECF factors (3%). MiST2 is updated monthly and is freely available for academic research at http://mistdb.com.

Keywords database; signal transduction; gene regulation

Theoretical design study for new β-lactamase inhibitors

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Clavulanic acid (CA, Figure) is produced by Streptomyces clavuligerus and was reported for the first time by Brown and coworkers. CA is a β-lactam compound that consists of a β-lactam ring condensed to an oxazolidin ring, it has a basic clavam structure and a 2R, 5R characteristic stereochemistry, which is essential for biological activity. CA is a strong inhibitor of β-lactamase enzymes produced by penicillins/cephalosporin-resistant bacteria. CA inhibits most class A β-lactamas, has low activity against class C cephalosporinases and is inactive against class B Zn2+ metalloenzymes.

There are also two other β-lactamase inhibitors in clinical use: Tazobactam (T, Figure) and Sulbactam (S, Figure), which are of synthetic origin. Both are structurally similar, Tazobactam has a triazol group substituent on the five membered ring, while on Sulbactam there is a β-methyl group.

The combination of CA and amoxicillin is the most successful example of a formulation containing a β-lactamase inhibitor and a broad spectrum β-lactam antibiotic. This formulation is available in Europe as Augentin® (DSM Anti Infectives – Delft, Holland) and in Brazil as Clavulin® (Smith-Kline-Beecham Laboratory – Rio de Janeiro). In addition there are other formulations such as Timentin, which combines CA and ticarcillin. Tazobactam and Sulbactam also can be found in a commercial formulation available as Unasyn (ampicillin/sulbactam) and Zosyn (piperacillin/tazobactam), respectively.

The main purpose of this study is to evaluate the molecular and electronic properties of various proposed β-lactamase inhibitors which structure search paining was based on the structures of Clavulanic Acid, Tazobactam and Sulbactam, the β-lactamase inhibitors in clinical use. The modified structures were optimized by a Quantum Chemical ab initio method to calculate the chemical and molecular properties of the compounds, by applying hierarchical methodology for the geometry optimization. Further, various indicators of interest were evaluated with the ab initio HF method implemented with Pople's basis set 6-31G (d,p), the calculations were performed with the GAUSSIAN 03 program. The properties evaluated were the energies of frontier molecular orbitals (εHOMO and εLUMO), the GAP HOMO-LUMO, molecular hardness (η) and hyperpolarizability (β).

The Quantum Chemical properties calculated indicate that some of the proposed structures analyzed may potentially improve the biological activity. These structures may be interesting synthetic alternatives to test as β-lactamase inhibitors.

Key words: β-lactamase inhibitor, structural modification, frontier molecular orbital, GAP, molecular hardness, hyperpolarizability.
Two-dimensional toxicological screening by massive parallel microcultivation in nanoliter fluid segment sequences

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The technique of micro segmented flow [1-3] was applied for the determination of high-resolved two-dimensional dose-response relations for microorganisms. Therefore, cell suspensions were automatically aliquoted in about 400 to 500 single cultivation volumes of 0.5 μL volume separated by a water-immiscible inert liquid. Two-dimensional, concentration spaces of effectors were realized by stepwise varied dosing in two injection channels by the application of PC-controlled syringe pumps. The quality control of fluid segments was realized by automated optical size and distance measurements. The addressing of the single points in the concentration space was checked by application of non-toxic dyes and microphotometric measurements of the single fluid segments. Micro flow-through photometers and a micro flow-through fluorometer were applied for the determination of cell density, endogeneous autofluorescence and pH.

E. coli, Saccharomices cerevisiae and Chlorella vulgaris were chosen as model organisms for the microtoxicological studies. Their growth behaviour and physiological activity were monitored [4] by micro-turbidometric measurements (cell density) and by fluorescence measurements. The effects of dinitrophenol, dichlorophenol, gold nanoparticles and silver nanoparticles and combinations of them were investigated.

High-resolved dose/response functions for all effectors and all organisms were obtained. In the two-dimensional studies different types of combination effects were found. The method allowed to distinguish between simple additive, lower and stronger synergistic behaviour. The two-dimensional map reflects the determination of critical concentrations and types of synergistic effects. In addition, it shows the quantification of influence of one effector on the effect of a second effector. These maps clearly indicate different types of cell response in different regions of the two-dimensional concentration spaces and allow to distinguish between different toxic combination effects. A complex behaviour was found, for example, for the effect of gold nanoparticles (GNP) and 2,4-dinitrophenol (DNP) (Fig. 2). GPNs are tolerated up to an Au equivalent concentration of about 1.4 μmol/L in case of low DNP concentration. But, a low increase in DNP concentration leads to a reduction of growth rate by application of lower GNP concentrations. The toxic threshold is effected by GNP as well as DNP. It is steep at high GNP and lower and mediate DNP concentrations, but becomes smooth at lower GNP concentration. The obtained results demonstrate that micro segmented flow is a powerful technique for gaining toxicological data from complex systems. It has the potential for analysis in higher dimensional concentration spaces and for ultraminiaturized ecological investigations.

Keywords: Toxicology, E. coli, Chlorella, nanoparticles, phenols, cell cultivation, segmented flow, microfluidics

Unraveling hyphal heterogeneity in Aspergillus niger by genome-wide expression analysis of single hyphae

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Mycelial fungi use the hyphae growing at their apices to colonize a substrate. These hyphae secrete enzymes that convert complex polymers into small molecules that can be taken up by the fungus to serve as nutrients. Previously it has been shown that proteins are only secreted by the limited number of growing hyphae within the mycelium. Over and above this, not every growing hypha secretes a particular protein. For instance, glucoamylase is secreted by growing hyphae at the periphery of a colony of Aspergillus niger but not by the growing hyphae in the central zone. This zonal heterogeneity can be explained by the availability of carbon source and, to a similar extent, by medium independent mechanisms. Subsequently, using GFP as a reporter, it has been shown that exploring hyphae at the periphery of A. niger are also heterogenic with respect to enzyme secretion; some hyphae strongly express the glucoamylase gene glaA, while others express this gene lowly. This was a surprising finding considering the fact that all hyphae were exposed to the same nutritional conditions. Apparently, a vegetative mycelium is more complex than generally assumed.

To establish whether hyphae differentiate into for instance secreting and non-secreting hyphae or whether several secretory types co-exist we want to perform a genome-wide expression analysis of hyphae highly and lowly expressing glaA. To this end, RNA will be extracted and amplified from both types of hyphae. We have set up protocols to collect the different hyphae by laser dissection using P.A.L.M. and to isolate RNA from 1-100 hyphae. QPCR has shown that we are able to extract RNA from even a single hypha. We have now started to amplify RNA to obtain sufficient amounts for hybridizations of whole genome arrays. Analyzing these arrays will help us unravel the mechanism behind hyphal heterogeneity.

Keywords: single cell analysis, heterogeneity, Aspergillus niger

This research is supported by the Dutch Technology Foundation STW, applied science division of NWO and the Technology Program of the Ministry of Economic Affairs.
Validation of serological test for diagnosing Helicobacter pylori infection in dyspeptic H. pylori culture positive children in Georgia

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Background: Serological tests for diagnosing Helicobacter pylori (H. pylori) infection are commonly employed but they vary in different population and require local validation. A pilot study conducted in Georgia in 2003 showed high prevalence (72%) of H. pylori in adult population ≥18, measured by a validated, point-of-care 13 C-urea breath test (Meretec Corporation, Lafayette, CO, USA). The study was aimed to determine sensitivity and specificity of ELISA test in dyspeptic subjects with positive microbiological culture results for H. pylori.

Methods: 132 dyspeptic previously untreated subjects aged 3-17 were selected randomly from a population survey. H. pylori was isolated from feces by bacteriological gold standard method. Blood samples were analyzed using Emory University (USA) experimental laboratory enzyme-linked immunosorbent assay (ELISA). Validity of the serological test was calculated with 95% CI.

Results: Of 132 subjects 41 (31.1%) were H. pylori microbiological culture positives. ELISA positive rate was 57.6%. A few subjects (8.3%) showed indeterminate results. The sensitivity and specificity of laboratory ELISA were 87.8% and 56% respectively. PPV composed 46.7% and NPV - 89.5%. Accuracy of the test was determined as 65.9%.

Conclusion: ELISA test results showed high sensitivity for H. pylori in children. As for specificity, on one hand, indeterminate results may impaired the study results in general and specificity of the test, in particular that should be considered in clinical appraisal, on the other hand. ELISA test results may be related to the geographical variations of the circulated H. pylori strains that itself requires further study and perhaps the development of ELISA test-kit using native strains.

Viscosity assessment in yeast vacuoles by Brownian motion of polyphosphate complexes

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In the vacuoles of the cells of the yeast Saccharomyces cerevisiae, at some cultivation conditions, optically dense vividly moving particles of approx. 0.5/541m size may appear. For their movement they were called “dancing bodies”. “Dancing bodies” were shown to be insoluble polyophosphate complexes (IPC). How and why they are moving is not known, although it was suggested to be Brownian motion. If it is proven to be Brownian motion, it must obey the Einstein-Smoluchowski equation, which for two-dimentional movement is the following:

\[ \langle s^2 \rangle = 4 \eta T t / 3 D, \]

where \( \langle s^2 \rangle \) – the average of the square of displacement; \( \eta \) – the Boltzmann constant; \( T \) – the thermodynamic temperature; \( t \) – the elapsed time; \( \eta \) – the viscosity; \( D \) – the diameter of the particle.

A peculiar feature of the “dancing bodies” is that, upon staining of the cells by 4’,6-diamidino-2-phenylindole (DAPI), a fluorescent dye for nucleic acids, they have a bright yellow fluorescent color (\( \lambda_{\text{max}} = 526\text{nm} \)) while nuclei and mitochondria fluoresce blue (\( \lambda_{\text{max}} = 456\text{nm} \)). That makes IPC convenient to observe and to study by fluorescence microscopy. The aim of this work was to quantitatively characterize by fluorescence microscopy the IPC movement in the vacuoles of the S. cerevisiae cells and to evaluate the viscosity in the vacuoles using the obtained data.

Along with the S. cerevisiae cells, fluoresceinisothyocyanate (FITC)-labeled latex microspheres of 2.1/541m and 3.1/541m diameter in water were also used in this study as a model system. Locations and displacements of the IPC and of the microspheres were determined on fluorescence microscopy images obtained by series of 8 shots at 0.43sec intervals by a Sony DSC-V3 digital camera. The images were analysed in retrospect using ImageJ, a computer image analysis software (National Institute of Health, USA).

The records of the IPC locations in the cells have shown that their movements were random/chaotic. So, the IPC “dancing” was Brownian motion indeed. Because the IPC movements were in the 3-D space, their fluorescence intensities changed or even vanished as they went out of the objective lense focal plane. This posed a problem in measurement of their 2-D displacements. On FITC-labeled latex microspheres a criterion for measuring the 2-D displacements of the fluorescing particles was developed. According to it, only the displacements for the particles, whose fluorescence intensities in a series deviated from the maximal no more than 15%, could be used for viscosity assessment by equation (1). Also, on microspheres, a method of the particle size determination by its fluorescence profile measurements was developed.

For four yeast cells, the sizes and the average displacements of their IPC were determined using methodology developed on latex microspheres. The viscosity values in the vacuoles of the four cells, as assessed using these data and the equation (1), was found to be of (in cP): 2.16 ± 0.60; 2.52 ± 0.63; 3.32 ± 0.9; 11.3 ± 1.7. The first three viscosity values correspond to the viscosities of the 30 - 40% glycerol solutions. The viscosity value of 11.3 ± 1.7 cP could be an overestimation caused by the restrictions of the IPC movements due to the peculiarities of the vacuole structure and volume in this particular cell. This conclusion was supported by the comparative analysis of the Brownian motion trajectories in the cells.

CONCLUSIONS
1. A new approach has been developed for assessing the intracellular Brownian movement characteristics and for the size determination of the fluorescing particles using fluorescence microscopy combined with computer image analysis.
2. Evaluation of the viscosity in the vacuoles of the S. cerevisiae cells using the developed methodology for IPC gave the values corresponding to the viscosities of the 30 - 40% glycerol solutions.

Keywords yeast; Brownian motion; vacuolar viscosity; polyphosphate; fluorescence microscopy; computer image analysis
Activity of Fluoroquinolones on *Staphylococcus aureus* and *S. saprophyticus* strains: Post-antibiotic Effect (PAE)

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Gram-positive cocci are important causes of infection both in the community and in the hospital, with repercussions on mortality and increased economic costs. The treatment of these infections is made difficult by the increasing emergence of multi-resistant organisms, primarily among Gram-positive cocci, such as methicillin-resistant. The activity *in vitro* of pefloxacin, ofloxacin, ciprofloxacin and norfloxacin against twenty eight clinical strains of *Staphylococcus aureus* and five strains of *S. saprophyticus*, respectively. The minimum inhibitory concentration (MIC) and postantibiotic effect (PAE) of pefloxacin, ofloxacin, norfloxacin and ciprofloxacin were evaluated *in vitro* against all strains of *S. aureus* and *S. saprophyticus*. All strains of *S. aureus* tested were susceptible to fluoroquinolones (42.85% to 64.28%), against norfloxacin and ofloxacin, respectively. The major resistance was observed to ciprofloxacin (32.14%), followed to norfloxacin (28.57%), ofloxacin (21.42%), and pefloxacin (17.87%). However, the strains of *S. saprophyticus* showed susceptibility to ciprofloxacin (60.00%), and similar results to norfloxacin, ofloxacin , and pefloxacin (40.00%). And was observed major the resistance to norfloxacin (40.00%), and similar results to pefloxacin and ciprofloxacin (20.00%), and no resistance to ofloxacin. The minimum inhibitory concentrations (MIC) was evaluated and indicated those strains are sensitive to fluoroquinolones (0.125 to 1.0 mg/L).

The bactericidal effect of fluoroquinolones was observed 0.25 to 2.0 mg/L for all strains of *S. aureus* and *S. saprophyticus*. The exposition to fluoroquinolones showed a great reduction of the maximum velocity (μMax-1) of growth of the bacteria, in special to ofloxacin and ciprofloxacin, after 2h of contact with the antibiotics. The PAE was studied in 6 strains by exposure of bacteria to pefloxacin, norfloxacin, ofloxacin and ciprofloxacin at 8 times minimum inhibitory concentration (MIC) for 2 h. Regrowth was determined by measuring the viable counts after drug removal by a 103 dilution procedure. PAEs increased as a function of concentration and exposure time. The mean duration of PAEs varied between 1.7 and 2.1h, showing the following order: ofloxacin>pefloxacin>ciprofloxacin>norfloxacin. These results are encouraging since fluoroquinolones have a possible role in the clinical treatment of *S. aureus* and *S. saprophyticus* infections, and the strong PAE caused by quinolones may contribute to the *in vivo* efficacy of these drugs. Those results explained the mechanisms of resistance of the antibiotics against *Staphylococcus* strains.

**Key words:** Fluoroquinolones, *Staphylococcus aureus*, *S. saprophyticus*, Effect Post-antibiotic.

Supported by CNPq, CAPES and FACEPE.

An efficient molecular typing assay for *Alternaria* spp. isolates

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Fungi are ubiquitous in the environment and are frequently isolated from household dust, soil, and decaying vegetable matter, including food and feed. Likewise, on animal mycological diagnosis, it is often observed that the detection of the relevant pathogens is disturbed by the faster development of many saprophytic contaminant fungi, such as *Cladosporium* and *Aspergillus* species, among others.

In previous studies, more than 1500 skin scrapings and hair samples obtained from dogs, cats and seals with skin lesions, were subjected to dermatophytes culture-dependent diagnosis. Concomitantly, the biological samples were also examined for the presence of saprophytic fungi. It is noteworthy that more than two thirds of the samples revealed the presence of known saprophytic contaminant fungi, being the *Alternaria* genus one of the most often found.

The genus *Alternaria* includes nearly 100 species of dematiaceous hyphomycetes that occur worldwide in a variety of habitats. Characteristics of the genus included the production of chains of dark-coloured multicelled conidia with longitudinal and transverse septa and a beak or tapering apical cells. Within the genus *Alternaria*, species are defined primarily upon conidium characteristics, among other sometimes ambiguous micromorphological features, leading to some confusion surrounding the conventional taxonomy of these fungi and definition of clear species and groups inside the genus.

We have been investigating molecular-based approaches for the fast and effective typing and identification of *Alternaria* spp. isolates. In this work we optimized a PCR typing assay for these fungi using 13 isolates from dogs, 13 isolates from cats and 9 isolates from seals. The assay is based on the amplification of polymorphic regions of genomic DNA using the microsatellite primer (GACA). This technique provides a fast, reliable and reproducible measure of genetic relatedness among closely related taxa and is especially useful as a first approach to species delineation in studies that involve large numbers of strains. Usually, conspecific strains display DNA banding patterns (fingerprints) with high overall similarity and give rise to well-defined clusters in dendrograms based on numerical analysis. Further taxonomic decisions on species boundaries must be normally based on selected gene sequencing of representative strains from each cluster of the microsatellite-PCR dendrogram.

**Keywords:** Alternaria, molecular typing.
Anatomopathological and mycological findings in two wild seagulls (Larus sp) infected with Aspergillus fumigatus

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Brooder pneumonia, “asper” mycosis or mycotic pneumonia, is a respiratory tract infection caused by fungi of the genus Aspergillus. Aspergillus fumigatus is the primary responsible for infections in wild birds with a compromised immune system. Predisposing factors such as migratory stress, malnutrition and primary infectious disease may play a role in the onset of the disease. In winter 2008, two seagulls (Larus spp) were found dead showing lesions of granulomatous pneumonia and thickening of the air sacs mainly due to the presence of grey coalescing plaques. Tissue samples were collected for histopathological and mycological analysis. Histopathological lesions were suggestive of severe pulmonary aspergillosis. Isolates of A. fumigatus were identified by cultural, morphological and microscopic characterization. This is the first reference of an Aspergillosis associated mortality in seagulls inhabitants of the Portugal coast.

Keywords: Aspergillosis; Aspergillus fumigatus; Seagull (Larus spp).

Anti-herpes simplex virus activity of a medicinal plant

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A Thai medicinal plant, Drymaria diandra, extracted with dichloromethane (DD) and methanol (DM) was used to investigate the presence of antiviral activity against herpes simplex virus type 1 (HSV-1) in Green Monkey Kidney (GMK) or Vero cells. Cytotoxicity tests performed by the MTT assay revealed that CD50 values of the DD and DM extracts were 90±2 and 631±32 μg/ml, respectively. In all experiments, non-toxic concentrations of the plant extracts were used to test the inhibitory effect on various steps of HSV-1 life cycle. By plaque reduction assay, HSV-1 replication was inhibited by the DD and DM extracts with the 50% effective dose (ED50) of 21±4 and 47±13 μg/ml, respectively. The DM extract inhibited HSV-1 attachment and penetration, and protein synthesis, better than the DD extract. Slot blot hybridization revealed an inhibition of about 30% of HSV-1 DNA replication by the DD extract, whereas the DM extract showed about 60% inhibition. Western blots with anti-HSV-1 polyclonal antibody showed that both DD and DM extracts had an inhibitory effect on many HSV-1 proteins, particularly those at 45-47 kDa molecular weight. The HSV-1 UL41-encoded virion host shutoff (vhs) protein, is responsible for massive degradation of mRNAs shortly after infection. However, Northern blot analysis showed that vhs RNase activity was only modestly affected by the plant extracts. Interestingly, after infected cells were treated with either DD or DM extract, real-time PCR of reverse-transcribed HSV-1 DNA polymerase (UL30) transcripts demonstrated a dramatic reduction in the pol transcript to about 1-2% of the control. UL30 and its processivity subunit UL42 are both required for HSV-1 replication. Western blot analysis showed that the DM extract, but not the DD extract, had an inhibitory effect on the UL42 protein. In infected cells, the HSV-1 ICP0 activates transcription of many viral and cellular genes, and is essential for reactivation of latency and viral replication. In contrast, Western blot analysis showed that the DD extract, but not the DM extract, had inhibitory effect on the ICP0 protein. Our results demonstrate that the D. diandra extracts have specific inhibitory effects on multiple steps in HSV life cycle, most dramatically upon replication. Our recent findings suggest that a number of alkaloid and flavonoid compounds may be involved. We believe these molecules represent a new class of anti-HSV drugs.

Keywords herpes simplex virus; anti-viral; plant
Antibiotics processed by Supercritical Fluids: antibacterial activity assessment

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The design of pharmaceutical preparations in micro- and nanopaticulate forms has emerged as an innovative strategy to control and modify the release of drug formulations, offering an effective way to increase its bioavailability, efficacy and specificity. Methodologies based on Supercritical Fluids (SCF) features advantages in terms of experimental versatility, environment protection and peculiar processing conditions. An example of improved drug characteristics through SCF expertise can be offered by sustained and controlled release of antibiotics entrapped in polymeric micro- and nanoparticles of various formulations.

Although some authors have reported the processing of antibiotics by supercritical fluids, the impact of supercritical conditions on the bioactivity of antibiotics has not been addressed yet. In this work, we report the antibacterial activity of antibiotics processed using potentially harsh supercritical fluid conditions. The antibiotics were exposed to Supercritical Carbon Dioxide (SC-CO2) at 40ºC covering two pressure values (80 and 120 bar) at several exposure times (4h, 24h, 48h). Three antibiotics were also processed into nanoparticles by intense atomization using Supercritical Enhanced Atomization (SEA). The influence of different preparation parameters (supercritical fluid, pressure, temperature, exposure time) on antibiotics integrity and microbiological stability was carried out. The antibiotics evaluated in plain form were nalidixic acid, cefaclor, cefazidime, claritromycin, tobramycin, streptomycin, eritromycin, tigecyclin, vancomycin and minocycline. The antibiotics processed into nanoparticle form were tobramycin, vancomycin and minocycline. The antibacterial activity was performed by the Mueller-Hinton agar diffusion method against a collection of reference antibiotic-susceptible isolates Escherichia coli ATCC 25922, Pseudomonas aeruginosa ATCC 27853, Staphylococcus aureus ATCC 29213 and resistant clinical isolates. These isolates are nosocomial strains, identified from different Portuguese hospitals and producing multiresistant mechanisms, namely: Klebsiella pneumoniae producing CTX-M-15 beta lactamase; Pseudomonas aeruginosa producing a metallo beta lactamase IMP-5; Escherichia coli with a class 1 integron carrying the blaCTX-M-9 gene; Acinetobacter baumannii, the European clone II, producing an oxacillinase OXA-40 and meticillin resistant Staphylococcus aureus (MRSA).

The antibiotics under the above described physicochemical conditions, as well as the antibiotic nanoparticles, were successfully evaluated for their microbiological activity against reference and multidrug resistant isolates. The results have evidenced the preservation of the antimicrobial characteristics for all studied antibiotics, a crucial property for further technological development and clinical application, which could attest the use of the supercritical methodology to process the mentioned nanoparticles.

Keywords: multidrug resistance; supercritical fluids; nanoparticles

Antimicrobial activity of Thymus vulgaris, Matricaria chamomilla, Croton lechleri, Caléndula officinalis L., Julliana adstringens Schl against periodontopathogens microorganisms

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Periodontal disease is a chronic inflammatory disease of the periodontium that leads to erosion of the attachment apparatus and supporting bone for teeth and is one of the most frequently occurring infectious diseases in humans. Porphyromonas gingivalis and Aggregatibacter actinomycetemcomitans (formerly Actinobacillus actinomycetemcomitans), are the principal microorganisms implicated in the process. Due to the increased drug resistance and the secondary effects of some antibiotics and mouthwashes like chlorhexidine, used in the treatment of periodontitis, justify the use of natural sources like the plant extracts as other alternative of dental treatment.

Aqueous extracts from various plants are used in Mexican traditional medicine on some microorganisms associated with different infections.

The antimicrobial properties of Thymus vulgaris (Tomillo), Matricaria chamomilla (Manzanilla), Croton lechleri (Sangre de Dragu), Caléndula officinalis L. (Caléndula), Julliana adstringens Schl (Cuachalalate) were evaluated in vitro against Porphyromonas gingivalis ATCC 33277 and Aggregatibacter actinomycetemcomitans ATCC 43715. Minimum inhibitory concentrations (MIC) is the lowest concentration of extracts to inhibit the growth of microorganisms. Minimum bacterial concentrations (MBC) is the lowest concentration of a drug that results in more than 99.9% killing of the bacteria being tested. MIC for each extract was determined by the microdilution method with dilutions ranging from 0.4% to 50%. Minimal Bactericide Concentration (MBC) was determined by agar diffusion and evaluated by absence of microorganisms on plate agar after incubation for 24h at 37ºC. Our results showed that Julliana adstringens Schl exhibited strong bacterial activity against P. gingivalis and A. actinomycetemcomitans at a concentration of 37 mg/ml, followed by Thymus vulgaris at a concentration of 62.5 mg/ml. Matricaria chamomilla and Calendula officinalis L. need higher concentrations (250 mg/ml.)

The five extracts inhibited the growth of bacteria at concentrations ranging from 37 mg/ml to 250 mg/ml.

Keywords: Thymus vulgaris, Matricaria chamomilla, Croton lechleri, Calendula officinalis L., Julliana adstringens Schl., antimicrobial activity
Assessment of mutagenic and carcinogenic of PTFE (Ames test)
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Introduction and objectives: The high quality and sanicity of ingredients as well as what we use daily are very important. The necessity of analysis the polytetrafluoroethylene composition, lead us to start this research. The present study, therefore, aims to evaluate mutagenicity and carcinogenicity effect of the compositions.

Material and Methods: The method is based on Ames test applying Salmonella typhimurium strains TA100, TA98 which received directly from Professor Ames. In the first stage, these strains for property purity of mutation were confirmed. Then, polytetrafluoroethylene compositions have been added separately to minimal agar medium contain fresh overnight culture (TA100, TA98) and compared to positive control (index of mutagen sodium azide and bacteria) and negative control (index of distilled water and bacteria). In the other stage rat liver tissues microsomes produced under sterile condition had been added separately to minimal agar medium with polytetrafluoroethylene compositions and it was measured the effect of the carcinogen material and bacteria status affected by reverse mutation and histidin produced.

Results: There was a specific mutation in the histidine synthesis gene that made these strains (TA100, TA98) become an external source of histidine for theirs growth. These strains upon exposure to an external mutagen undergo a reverse mutagenicity in theirs mutated histidine synthesis operon and these strains then can grow in a histidine free medium. In this research considering to colony numbers and comparison with negative control index containing several colonies produced by spontaneous mutation approved materials mutagenicity and compared.

Conclusion: This critical case makes us have more care and control on the mechanism of polytetrafluoroethylene compositions production and use.

Key word: PTFE, Ames test, TA100, TA98

Beneficial effects of HIV peptidase inhibitors on Fonsecaea pedrosoi: promising compounds to arrest key fungal biological processes and virulence
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Fonsecaea pedrosoi is the principal etiologic agent of chromoblastomycosis, a fungal disease whose pathogenic events are poorly understood. Chromoblastomycosis is a chronic, supplicative and progressive mycosis of the skin and subcutaneous tissues. Chromoblastomycosis lesions are recalcitrant and extremely difficult to eradicate. Chemotherapy, surgical excision and/or cryosurgery have been used throughout the years, but an effective treatment for chromoblastomycosis has not yet been established. Current therapy for chromoblastomycosis is suboptimal due to toxicity of the available therapeutic agents and the emergence of drug resistance. Compounding these problems is the fact that endemic countries and regions are economically poor. Consequently, the search for new anti-F. pedrosoi targets and anti-F. pedrosoi strategies is a critical task. In this sense, proteolytic enzymes are considered potential molecules for development of new antifungal agents. Peptidases participate in several physiological and pathological processes in different cell types. In pathogenic fungi, this class of hydrolytic enzymes directly acts in different steps of the microorganism-host interplay, being considered as virulence factor. Considering all these facts together, we have conducted a study to investigate the direct effect of four different human immunodeficiency virus (HIV) peptidase inhibitors (PIs) (indinavir, saquinavir, ritonavir and nelfinavir), commonly used in highly active anti-retroviral therapy (HAART), on the F. pedrosoi conidial secreted asparagine peptidase activity, growth ability, ultrastructure and interaction of this human pathogen with distinct animal cell lineages in vitro. The possible synergistic effect between PI and antifungal compounds was also available. All the HIV-PIs impaired the acidic conidal-derived peptidase activity in a dose-dependent fashion, in which nelfinavir produced the best inhibitory effect. F. pedrosoi growth was also significantly reduced upon exposure to HIV-PIs, especially nelfinavir and saquinavir. HIV-PIs treatment caused profound changes in the conidial ultrastructure as shown by transmission electron microscopy, including invaginations in the cytoplasmic membrane and withdrawal of the cytoplasmic membrane from within the cell wall, disorder and detachment of the cell wall, enlargement of fungal cytoplasmic vacuoles, and abnormal cell division. The synergistic action on growth ability between nelfinavir and amphotericin B, when both were used at sub-inhibitory concentrations, was also observed. HIV-PIs reduced the adhesion and endocytosis indexes during the interaction between conidia and epithelial cells (CHO), fibroblasts or macrophages, in a cell type-dependent manner. Moreover, HIV-PIs interfered with the conidia into mycelia transformation when in contact with CHO and with the susceptibility killing by macrophage cells. Overall, by providing the first evidence that HIV-PIs directly affects F. pedrosoi development and virulence, these data add new insights on the wide-spectrum efficacy of HIV-PIs, further arguing for the potential chemotherapeutic targets for asparagine-type peptidase produced by this human fungal pathogen.

Financial Support: CNPq, FAPERJ, CAPES and FUJB

Keywords Fonsecaea pedrosoi, chromoblastomycosis, secreted aspartic peptidase; HIV asparagine peptidase inhibitors
Capsular Types of Haemophilus influenzae Isolated from CSF of Children with Meningitis and Pneumonia, in Iran

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The Haemophilus influenzae (Hi) isolates were isolated from CSF of children with meningitis and pneumonia like symptoms admitted to Tehran hospitals. The capsule genes of Hi were analyzed and compared with serologic capsule typing. The isolates were tested for their X and V factor requirement, catalase test, Quellung reaction, and biotyped, serotyped by slide agglutination serotyping (SAST). Based on these tests, seventy five invasive Hi species were isolated and identified from 102 serum and CSF samples of children below 5 years of age. The Hi isolates were then evaluated using PCR capsule typing using primers specific for the types a-f cap genes and bexA genes. Discrepancies were found between SAST and PCR capsule typing and three of the samples appearing negative by cultural reactions were tested positive by PCR. Moreover, 14 isolates appearing encapsulated by SAST were NTHi by PCR, 7 NTHi by SAST were encapsulated by PCR, 5 encapsulated by SAST were a different capsule type by PCR and 2 encapsulated by SAST were capsule-deficient Hib variants (Hib-minus). Overall, the results of SAST and PCR capsule typing for twenty eight of (37%) isolates were not in accordance. The sensitivity, specificity, and accuracy index of PCR capsule typing was 100%, 90.2% and 97% respectively.

Keywords: H. influenzae, encapsulated, nontypeable, serotyping, capsule typing.

Chemical and pharmacological study of Brazilian marine Streptomyces.

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Natural Products Chemistry has resulted in the discovery of hitherto unknown organic compounds; many of them have been used in pigments, insecticides, drugs, etc. Studies of plants and terrestrial microorganisms have been shown to be extremely important as they generate many economically important organic compounds. For example, 25% of the anticancer drugs in clinical use and approximately 25% of semi-synthetic drugs are derived from natural products. The main goal of this work was to present the chemical and pharmacological study of the marine microorganisms Streptomyces acrymicini and Streptomyces cebimarensis collected in São Sebastião, north coast of São Paulo state, Brazil. The growth broth was centrifuged to eliminate the cells and the “cell free” solution was extracted with ethyl acetate and butanol, obtaining three crude extracts for each microorganism. The organic extracts obtained were tested in cytotoxic and antitumor bioassays using SF-295 (Central Nervous System) and HCT-8 (Colon) strains (using the MTT method), and also antibacterial and antifungal tests. The crude extracts were separated/purified using bioassay guided chromatographic separation with the aim of isolating the bioactive secondary metabolites responsible for the antitumor, cytotoxic, antibacterial and antifungal activities as shown in schemes 1 and 2. From the bioassay guided chromatographic separation were obtained, up to the present moment, six bioactive compounds with structures that are currently being determined by 1-D and 2-D NMR spectroscopy and mass spectrometry.

Keywords: marine Streptomyces, chromatography, cytotoxic activity, antitumor activity, antibacterial activity, antifungal activity.
Colicin E1 production is associated with bacteriocin multiproducer strains in uropathogenic Escherichia coli

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The incidence of 29 bacteriocin types were tested in a group of 377 E. coli strains isolated from urinary tract infections (UTI) and in 413 control strains isolated from feces of patients without bacterial gut infection. Sixteen and 20 individual bacteriocin types were found in the UTI and control strains, respectively. The spectrum and frequency of individual bacteriocin types in the UTI strains was very similar to the control strains, probably reflecting the origin of UTI strains in the human gut. In the group of E. coli UTI strains, 159 bacteriocin producing strains (42.2%) were identified being not significantly different from 199 bacteriocin producers in the control group (48.2%). Producers with 3 and more identified bacteriocin types were more frequent in the UTI group (14.1% compared to 14.1% in control, p = 0.005). In the UTI strains, there is a marked increase of strains producing colicin E1 when compared to control (14.3% and 6.8%, respectively, p = 0.002). Producers with 3 and more identified bacteriocin types were more frequent in the UTI group (25.8% compared to 14.1% in control, p = 0.005). In triple producers and multiproducers, this association was very strong (p < 0.0001). Producer strains with the combination of bacteriocins Ia, E1 and mV were more frequent in the UTI group when compared to control (7.6% and 1.5%, respectively, p = 0.004). In addition, decreased frequency of colicin Ib producers in the UTI group of strains (3.1% and 7.5%, respectively, p = 0.02) was found. Despite similarities in the spectra and in frequencies of individual bacteriocin types in both groups, there is a tendency in the UTI strains to possess additional genetic determinants and to synthesize colicin E1.

Keywords: bacteriocin; colicin; microcin; uropathogenic E. coli strain

Comparative Study on the Sensitivity of Daptomycin Against Vancomycin by MRSA from Hospital

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The dilemma of empirical treatment of infections caused by gram-positive has become more frequent. The delay in appropriate antibiotic therapy may increase the length of stay in hospital patients with MRSA septicemia. Turn strains of MRSA have become progressively less sensitive over the years. High MICs reduce the effectiveness of vancomycin in MRSA infections. MICs are also considered high predictors of mortality in the treatment with vancomycin. The daptomycin, a natural cyclic lipopeptide, with rapid bactericidal activity against Gram-positive pathogens, is an alternative treatment for complicated infections such as septicemia and endocarditis caused by MRSA. The aim of this study is to evaluate the susceptibility of daptomycin compared with vancomycin in samples of MRSA (Staphylococcus aureus methicillin-resistant) isolated in hospital. A total of 76 clinical samples of MRSA were studied and analyzed from patients in a public hospital in Recife, Brazil, between 2008 and 2009. Samples were obtained from blood, urine and secretion surgery. For the identification test was used conventional and automated methods. The susceptibility profile was carried out by E test, with ribbons of vancomycin and daptomycin, gradients performed, pre-defined and stable, that have a constant and optimum level of calcium, onto Mueller Hinton Agar standard, according to methodology recommended the CLSI. All strains of MRSA were susceptible to vancomycin, and detected a significant increase of MIC curve, but within the cutoff interpretive sensitivity set by CLSI. In relation to daptomycin, all the samples also showed sensitivity, with much lower MICs, ranging between 0.32 and 0.64 mg/mL. According to obtained results can be concluded that daptomycin showed significant improvements when compared to vancomycin, such as increased in vitro potency represented by lower MICs, and rapid bactericidal activity. Recent studies of pharmacokinetics and pharmacodynamics (PK-PD) demonstrate that daptomycin offers better coverage compared to vancomycin for the treatment of systemic infections caused by MRSA. To test the sensitivity of daptomycin should be considered the characteristics of the drug as the high molecular weight and the dependence of free calcium ions in physiological conditions.

Keywords: MRSA, Vancomycin, Daptomycin.
Conjugation of *Haemophilus Influenzae* type b capsular polysaccharide and tetanus toxoid using DMT-MM as activating agent

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*Haemophilus Influenzae* serotype b (Hib) is a capsulated bacterium that causes invasive infections, which the most frequent is meningitis. The capsular polysaccharide (PS) is the main factor of virulence, and as consequence, the main antigen for vaccines. However, PS are T-cell-independent antigens and their covalent linkage to a protein carrier converts them in a T-cell-dependent antigen making it efficient to induce protection in young children. The objective of this work is to develop a conjugation process between capsular polysaccharide of *H. influenzae* type b (polyribosil ribitol phosphate - PRP) and tetanus toxoid (TT) with a desired conjugation yield and suitable to scale-up. For this purpose we developed a method which the reaction sequence was summarized in Fig.1, employing DMT-MM (4-[4,6-dimethoxy-1,3,5-triazin-2-yl]-4-methylmorpholinium chloride) as activating agent in the last reactional step.

**Figure 1 - Reactional sequence**

The first reaction is the oxidation of native polysaccharide (PRP) with generation of the reactive aldehydes groups (PRP-Oxi). The reaction mixture was prepared mixing PRP (10mg/ml), NaOCl (10mM) in phosphate buffer 10mM pH 7 in the dark for 30 minutes and the reaction was stopped adding glycerol (10eq). PRP oxidation resulted in polysaccharide of lower size and the oxidation condition was established in order to result a PRP with molar mass about 50kDa and about 5 moles of aldehyde per mol of PRP. The second step is the reaction of aldehyde group with adipic acid dihydrazide (ADH) followed by reduction with NaBH₄. The PRP-Oxi (6 mg/ml) was mixed with adipic acid dihydrazide (10 eq) in phosphate buffer 10mM, pH 7 for 3 hours. The reaction condition was established to obtain the maximum of the aldehydes groups reacted with adipic acid dihydrazide. In the last step, the reaction between PRP-ADH and tetanus toxoid, the carboxyl groups of the protein must be activated to promote the reaction with polysaccharide. A soluble carbodiimide EDAC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride) is usually used for this purpose. In this work we have tested DMT-MM as activating reagent. The main advantage of DMT-MM over EDAC is that it is less susceptible to hydrolysis than EDAC. Besides, EDAC can generate undesired side groups by the structural rearrangement of the *N*-acylurea. The reaction of carboxyl groups with DMT-MM occurs by a SNAr mechanism forming a triazinyl ester and this active ester can react with nucleophiles like amine groups.

Comparison of the conjugation process using EDAC and DMT-MM as activating reagent showed a clear advantage to DMT-MM which resulted in a reaction yield twice higher than that obtained with EDAC. The final product PRP-TT conjugate is purified by gel filtration chromatography in Sephacryl S-400 or Phenyl-Sepharose chromatography. The advantage of the use of Phenyl Sepharose is the recovery of the free PRP.

Supported by FAPESP, CNPq and Fundação Butantan

**Keywords:** *Haemophilus Influenzae* capsular polysaccharide; tetanus toxoid; DMT-MM; conjugate vaccine.

Convergent acquisition of bacterial Antibiotic resistance determinants from experimental animals

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The pattern of acquiring antibiotic resistance genes (ABRG) among microbial flora in experimental animals is strengthened by the intensive overuse of antibiotics (AB's). A survey of antibiotic resistance bacteria was carried out to characterize the antibiotic resistance in experimental animal litter (EAL). One hundred and twenty isolates from EAL were isolated and screened for phenotypic resistance to eight antibiotics by the disk diffusion method. The distribution of species such as *Staphylococcus* (n=30), *Streptococcus* (n=30), *Micrococcus* (n=30), *E.Coli* (n=15), *Salmonella* (n=10) and *Aeromonas* (n=5) showed a remarkable resistance against antibiotics. Extent of resistance against antibiotics in isolated strains was investigated by Minimal inhibitory concentration (MIC). Resistance rate of isolated strain was in the following order *Salmonella > E.coli > Aeromonas*. 16S rRNA analyses were carried out to find out the dominant antibiotic resistant groups in EAL. The data can be used as a baseline to determine antibiotic resistance pattern and in evaluating the pathogenic microbial community which has an high lethal impact on humans and livestock.

**Keywords** Antibiotic resistance genes; experimental animal litter, Minimum Inhibitory Concentration, 16S rRNA

*¹and ²Author have equal credits

*²We would like to thank FEMS for supporting us to attend BioMicroWorld 2009, Lisbon, Portugal, through FEMS meeting attendance grant.*
Degradation of Sgs1 in response to rapamycin treatment in yeast *Saccharomyces cerevisiae*

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In yeast *Saccharomyces cerevisiae*, rapamycin an immunosuppressant inhibits the TOR (Target of Rapamycin) complex, homologous of mTOR in mammalian cells. It triggers events that mimic the effect of nutrient starvation including inhibition of ribosome biogenesis, protein translation and inducing autophagy and G0 entry. TOR proteins integrate signals from growth factors nutrients, stress and cellular energy levels to control cell growth.

Rdn1 is a cis/trans prolyl isomerase that required signalling responses to the immunosuppressant rapamycin and mutant devoid of rdn1 display striking resistance to the drug.

Preliminary data revealed that this effect was blocked by removing the helicase Sgs1. Thus our objective was to investigate what is the link between Rdn1 and Sgs1. Sgs1 is a nuclear DNA helicase of the RecQ family involved in genome integrity maintenance. RecQ family is conserved from bacteria to humans; Sgs1 is a homolog of human BLM and WRN proteins implicated in Bloom and Werner syndromes. Deletion of SGS1 leads to diverse phenotypes including sensitivity to genotoxic agents, hyper-recombination, chromosome missegregation, and meiotic defects. Mutations in the SGS1 gene lead to defects similar to those seen in human cells from the RecQ family disorders.

Sgs1 mutant is sensitive to rapamycin. Significantly, deletion of Sgs1 in the Rdn1 mutant causes the mutant to no longer show resistance to rapamycin. Reintroduction of Sgs1 will restore to the rdn1 mutant resistance to rapamycin. The sensitivity to Rapamycin remains at the same level even if Sgs1 is over expressed in the wild type strain and Rdn1 mutant. Sgs1 may belongs to the pathway that signals stress caused by rapamycin.

Rapamycin treatment induces rapid degradation of Sgs1 and this degradation is dependent on Rdn1 function. It has recently been demonstrated that Rdn1 is required to isomerize the C-terminal domain of RNA polymerase II and caused its release from the chromatin for degradation. Based on this observation, Rdn1 could most probably alter the structure of Sgs1 then it gets degraded in response to rapamycin.

Sgs1 accumulates in Rdn1 mutant after rapamycin treatment as detected by immunofluorescent analysis. This finding reveals a lower level resistance after rapamycin treatment in parent strain and accumulation in Rdn1 mutant. These observations support that Rdn1 is necessary to degrade Sgs1 in the cell, but mechanisms of this degradation are not yet known.

Key words: yeast; Sgs1; Rdn1; rapamycin

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Determination of mode of action for novel synthetic antifungal agents using reversal assay method

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A narrow range of proteins or metabolic pathways are targeted by the current available antifungal drugs and many fungi have gained complete resistance to the action of these drugs. Hence, the identification of new targets is of great importance. Many of antimicrobial agents have been discovered through a massive screening of natural or chemical libraries. However, the current interest of the pharmaceutical industry is understanding the mode of action of new antimalarial compounds.

A classical method of identifying the mode of action is reversal assay, which has been used in the present study. The basis of this process is to reverse biological activity of a drug by adding metabolic intermediates or final product of a given pathway. Here, we have used two synthetic antifungal compounds, A and B which are not structurally similar to current antifungal drugs. Synthetic medium RPMI 1640 enriched by 2% glucose and *Saccharomyces cerevisiae* genome integrity maintenance. RecQ family is conserved from bacteria to humans; Sgs1 is a homolog of human BLM and WRN proteins implicated in Bloom and Werner syndromes. Deletion of SGS1 leads to diverse phenotypes respectively. Reversal assays was performed in 96-well flat-bottomed microplates. Reversing concentration of each nucleotide, amino acid or TCA cycle intermediates, including sensitivity to genotoxic agents, hyper-recombination, chromosome missegregation, and meiotic defects. Mutations in the SGS1 gene lead to defects similar to those seen in human cells from the RecQ family disorders.

Reversal assay showed to be effective in identifying the inhibition sites and hence leading us to the mechanism of action. Our synthetic agents had effect on inhibition of nucleic acid pathway (compound A) and aliphatic amino acid synthesis and to a lesser extent on TCA cycle (compound B). Further study to identify the main target of each compound is underway.

Keywords antifungal; reversal assay; synthetic compound; mode of action
Development of Antibacterial Preparations Containing Fermented Products from Some Thai Herbs

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Fermented plant products are widely used in all part of Thailand. The products have been utilized in many application such as agricultural and environmental sciences, household products, food supplements and cosmetics. The objective of this research was to study antibacterial activities of crude extracts and of fermented products from some Thai herbs such as Kaempferia parviflora, Garcinia mangostana and Morinda citrifolia Linn., which were to be developed as effective topical and antibacterial preparations. The crude extracts were obtained by maceration of the dried herb powder with 95% ethanol, then evaporated to a concentrated crude extract. The fermented products were obtained by fermentation of dried plant, honey and water with Lactobacillus acidophilus for 4 months. They were filtrated though a 0.45 μm membrane filter and characterized for pH, density and appearance before further study. The fermented herb juices and crude extracts were then tested for antimicrobial activity to determine the MIC values against Staphylococcus aureus ATCC 25922, Pseudomonas aeruginosa ATCC 27853, Streptococcus β group A, Escherichia coli ATCC 25922 and Candida albicans ATCC 90028. It was found that the MIC value were 0.1263-1.0250 gm/ml for fermented products from each plant and were 0.0125-0.050013 gm/ml for their crude extracts. Selected topical preparations in the form of cream and gel containing each crude extract and each fermented product from Kaempferia parviflora and Garcinia mangostana in the concentration of 4 times of its MIC value were prepared and characterized in terms of appearance, pH and viscosity. Antibacterial activity as well as product stability under various tested conditions were also evaluated. The results showed that both creams containing the crude extract and fermented product from Kaempferia parviflora were the most stable and revealed effectiveness against S. aureus and Ps. aeruginosa, to an extent comparable to a gentamycin cream. Furthermore, they exhibited no skin irritation according to patch tests in 10 volunteers. None of the gel preparations were stable under some tested conditions but all showed good antibacterial activity after stability testing. It was concluded that fermented products and crude extracts from Kaempferia parviflora can be prepared as effective topical antibacterial cream and should be further investigated for clinical use.

Keywords: Fermented products, Thai herbs, antimicrobial activity, topical preparations

Effect of plants used in Mexican traditional medicine on Candida albicans biofilm formation

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Mexico has a great wealth of medicinal plants and it has been popular tradition to use these plants for scientific investigation, many of which deal with the antimicrobial properties of the plant extracts and their potential as a clinically relevant antimicrobial therapy. Recently there has been an unacceptably high increase in antifungal drug resistance, particularly in Candida albicans. C. albicans is an opportunistic pathogenic fungus and is the most frequent causative agent of candidiasis. Candidiasis is an increasing health threat to immune compromised individuals and infections are commonly associated with the formation of biofilms on the surfaces of biological and inert surfaces. Due to the increased drug resistance and lack of effective antifungals for the treatment of Candida infections, screening plant extracts for antimicrobial activity is a potential means of identifying new antifungal agents. The objective of these experiments is to determine if plant extracts from Thymus vulgaris, Croton lechleri, and Julliana adstringens Schl. are effective in the prevention and treatment of Candida albicans biofilm formation. Different parts of these plants were collected and extracted with solvents to obtain aqueous and organic extracts. These extracts were tested in vitro for their antifungal activity against C. albicans using a 96-well microtiter plate model of C. albicans biofilm formation and inhibition. This model is coupled with a colorimetric XTT-reduction assay in which metabolically active sessile cells reduce a tetrazolium salt to a water-soluble orange formazan compound, which can be quantified using a microtiter-plate reader. Our results showed that J. adstringens Schl. exhibited strong antifungal activities against C. albicans biofilm formation inhibiting biofilm formation by 88.25% at a concentration of 62.5 μg/mL, while T. vulgaris and C. lechleri had low activity against the formation of C. albicans biofilms. None of the plant extracts had inhibitory effects on preformed C. albicans biofilms.

Keywords: Thymus vulgaris, Croton lechleri, Julliana adstringens Schl., Candida albicans, antimicrobial activity, biofilm.
Effects of endocannabinoids and 3-dezaadenozine on the growth of free-living amoebas and their phagocytosis activity

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Free-living amoebas of the Acanthamoeba and Naegleria genus are responsible of severe brain infections of increasing prevalence, especially in immune compromised patients. In addition to numerous side effects such as seizures, nausea and vomiting, granulomatous amebic encephalitis is usually fatal to the patient for whom no efficient therapeutic treatment is available. It has been shown that cannabinoids such as ∆9-tetrahydrocannabinol (THC) inhibit the growth of the pathogenic amoeoba Naegleria fowleri in vitro. However, in vivo this cannabinoid also has immunosuppressive activities and it was shown to exacerbate brain infection by amoeba in animal models. At the opposite to THC, some endocannabinoids (N-arachidonolamines and 2-O-acyl glycerol) display an immunostimulatory effect, including stimulation of macrophages and microglial chemotaxis. The putative effects of endocannabinoids on free-living amoebas is not known. We thus tested different N-arachidonolamines and 2-O-acyl glycerol on the growth of free-living amoebas (Acanthamoeba castellanii, Williaertia magna and Hartmannella vermiformis) and their phagocytosis activity. The results show that N-arachidonolamine and 2-O-acyl glycerol (including N-arachidonolamine ethanolamine [anandamide] and 2-O-arachidonoyl glycerol) strongly inhibit in a dose dependent manner the growth of free-living amoebas. Phagocytosis activity was also reduced upon endocannabinoid treatment. Similar observations were made when using 2-arachidonoloyl ether, a non-hydrolysable structural analog to 2-O-arachidonyl glycerol, showing that 2-O-arachidonoyl glycerol per se rather than a catalytic product explains these effects.

We then tested 3-dezaadenosine, a known inhibitor of the phosphatidyethanolamine (PE) methylation pathway for biosynthesis of phosphatidylcholine (PC), the major phospholipids in protozoa. The impetus that phospholipid synthesis is a critical event in cell growth. We found that inhibition of the phosphatidethanolamine methylation pathway by 3-dezaadenosine induces growth arrest in all amoebic genus and that Willaertia magna, that displays a high PC/PE ratio when compared to other amoebaic genus, is particularly affected.

Keywords: Anandamide, 2-arachidonoyl glycerol

Ethnomedicinal survey of medicinal plant species used as remedy for HAV/HBV/HCV by the ethnic groups of Bangladesh

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Hepatitis A, Hepatitis B, and Hepatitis C are viruses (HAV/HBV/HCV), which causes HAV/HBV/HCV infections. HAV/HBV/HCV is one of the world’s most common infectious diseases. Infection can lead to severe liver disease, including chronic hepatitis, liver cirrhosis, and primary liver cancer. More than one million deaths per year are recorded due to HAV/HBV/HCV infections. HAV/HBV/HCV is the most common disease in Bangladesh. For the reason that a multicultural and ritual country Bangladesh is an alluvial plain land breaks the scenario by not only topographical but also human habitations. There are as many as forty two different ethnic groups living in Bangladesh. Each ethnic group has their own customs & traditions, religion, language, and culture. For cure of ailments, they rely on their own ethnic group healers who are experts in the knowledge and use of the medicinal plant species. There is little information on the medicinal plant species used by the ethnic groups of Bangladesh. The objective of this present study was to conduct an ethnomedicinal survey amongst the ethnic groups’ healers of the Khaisia, Tripura, Tanchangya, Manipuri, Chakma, and Garo ethnic groups to collect information on the medicinal plant species used to treat of HAV/HBV/HCV. In-depth information regarding medicinal plants type, preparation of medicine, ailments for which they are used, dosage, and side-effects if any, were obtained from the ethnic groups’ healers. Medicinal plant samples were collected and identified at the Bangladesh National Herbarium. Information on forty nine medicinal plant species was obtained, which were used by the ethnic groups’ healers to treat of HAV/HBV/HCV. The forty nine medicinal plant species are grown and consumed as summer, rainy, autumn, and winter seasons because there are six seasons in Bangladesh, distributed in forty eight genera and thirty seven families. The family Lamiaceae contained the highest number of medicinal plants (four species) followed by Rutaceae (three species), and Zingiberales (three species). Five families (Rubiacceae, Asteraceae, Acanthaceae, Anacardiaceae, and Lauraceae) had two medicinal plant species each. The remaining twenty nine medicinal plant species were distributed in twenty nine families. These medicinal plant species (with parts used given in parenthesis) included Aristolochia indica (seed, root), Centella asiatica (whole plant), Curcica papaya (seed, fruit), Cuscuta reflexa (whole plant), Achyranthes aspera (root, seed), Aegle marmelos (leaf, seed, fruit), Coprosara dulcis (whole plant), Ipomoea mauritian (tuber root), Lycopersicon esculentum (root, flower), Kalamhoe pinnata (whole plant), Eclipta alba (whole plant), Ficus racemosa (leaf, fruit), Citrus aueniferus (fruit, seed, leaf), Xanthesma spicata (fruit, seed, leaf), Zingiber officinale (tuber root), Acorus calamus (tuber root), Vitex negundo (whole plant), Punica granatum (leaf, fruit), Cinnamomum veram (bark), Randia duncetorum (whole plant), Morinda angustifolia (whole plant), Spondias dulcis (fruit, leaf), Terminalia arjuna (bark, fruit, Mucuna spicata (whole plant), Swintonia floribunda (fruit), Plantago major (seed), Svertia chirata (whole plant), Cinnamomum tamala (leaf), Nymphaea nouchali (whole plant), Dillenia indica (fruit, seed), Ipitys suaveolens (whole plant), Dancus carvot (tuber root), Aloe barbadensis (whole plant), Feronia elephantum (fruit, seed), Coccs nucifera (fruit, root), Saccharum officinarum (stem juice), Elettaria cardamomum (fruit), Syzygium cumini (fruit, seed), Momorica charantia (fruit, seed), and Ross damaecassus (root, flower). Since the ethnic groups of Bangladesh mostly does not have access to primary medical facilities, the above medicinal plant species can form the basis of treatment for viral diseases without resorting to costly urban visits or allopathic practitioners. A number of these medicinal plant species are becoming highly endangered. These medicinal plant species can form a useful source for scientific studies and isolation of active constituents to treat of HAV/HBV/HCV.

Keywords: Khaisia, Tripura, Tanchangya, Manipuri, Chakma, and Garo ethnic groups, HAV/HBV/HCV.
Functional analysis of the widely conserved cytoplasmic domain of Spa24 in the T3SS assembly

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Type III secretion systems (T3SSs) are central virulence factors of many Gram-negative bacteria. Their overall morphology is consisting of a cytoplasmic region, an inner- and outer-membrane section and an extracellular needle. In this study, we investigate the role in Ipas secretion of Spa24, Spa9 and Spa29, three predicted inner membrane proteins, widely conserved among all T3SS. We generated individual non-polar spa24, spa9 and spa29 mutants and studied their phenotypic aspects in vitro. Mutants lacking either of these proteins were unable to secrete Ipas proteins and to invade HeLa cells. Electron microscopy analysis revealed that the three mutants exhibit needleless secretons. Considering the existence of protein–protein interfaces, we investigated potential interactions through GST pull-down experiments. We identified three interacting partners, the Spa32 domain required for Spa24SD binding located between residues 73 and 154. Furthermore, we demonstrate by genetic and functional analyses that six specific residues of Spa24CD, conserved among Spa24 T3S orthologous, are crucial for Ipas secretion and interaction with some identified partners. We lastly showed that Spa9 interacts directly with both Spa40C and MxiA and that Spa29 interacts separately with MxiAC and MxiN. Taking together, our data suggest that Spa24 plays a crucial role as a crossroad inside the bacterium as a critical component for substrate specificity switching.

Genotypic variability in the sequence encoding SpaP and mut II of cariogenic Streptococcus mutans strains in saliva samples intra and inter family members

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Streptococcus mutans, the major etiological agent of dental caries in humans, possesses a variety of virulence traits that enable it to establish in oral cavity and initiate disease. Among them, the proteins antigen I/II, B, Psa, SpaP and bacteriocins (mutacins) may be associated to S. mutants pathogenicity and be critical for oral colonization. In the present study, the variability of genes encoding spaP and mutacins type II (mut II) in mothers and offspring of ten families (and among families) of San Nicolás de los Garza, N.L., Mexico was evaluated by qPCR, using the reference strain ATCC 700611 of S. mutans as positive control. Presence of these genes was studied and compared within each family and inter families. Samples were collected from saliva, plated onto mitis-salivarius agar and were incubated for 48 hours. DNA was then isolated by a High Pure PCR template preparation kit. The results showed at least 2 to 4 variants in the sequences encoding for spaP and (mut II) genes; such variations were observed intra and inter families. The DMF (decayed, missing, filled teeth) index among mothers and offspring was 12.7 and 2.7 respectively.

Keywords S. mutans; genotypic variability; mutacin; spaP; caries.
Genotyping of Human Papillomavirus in abnormal cervical samples from the North-Eastern Croatia

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Human papillomavirus (HPV) is one of the most common sexually transmitted diseases worldwide. Most HPV infections are cleared by the immune system and do not result in clinical complications. Clinical consequences in case of low-risk HPV infection consist of genital warts and mild Pap test abnormalities, while High-risk HPV infection (HR HPV), especially with oncogenic HPV types HPV 16 and HPV 18, include abnormal Pap results, low and high-grade cervical intraepithelial neoplasia and cervical cancer, for which Chlamydia trachomatis may sometimes be a co-factor. During three-year period we tested 1,194 clinical specimens for HR HPV; 249 urethral swabs from men who had sexual contact with an infected person and 945 cervical swabs with normal and abnormal cervical cytological diagnosis. All samples were collected in the Osijek-Baranya County Institute of Public Health and gynaecologist’s offices. Subsequently 100 HR HPV positive cervical samples with abnormal cervical cytological diagnosis were genotyped by Linear Array HPV Genotyping Test (Roche Diagnostics). The association between certain HPV genotype and cervical intraepithelial neoplasia was determined as well as mixed infections with four or more HPVs. The aim of the study was to determine the prevalence of HR HPV and age-related profiles of HR HPV infections in population of our county. The obtained data indicate that HPV 16 and HPV 18 are the most common HR HPV genotypes in the cervical specimens with abnormal cervical cytological diagnosis among women in Osijek-Baranya County as in most studies worldwide. Obtained results also determined mixed infection of four HR HPV with one or more low risk HPV. Our results revealed that cervical HR HPV infection is strongly related to age, with highest occurrence at the age 25-34, in association with increased number of sexual partners. The obtained results and further analyses in this ongoing project could be useful tool for clinical and epidemiological characterization of HPV in our community.

Keywords: sexually transmitted diseases, high-risk HPV, low-risk HPV, HPV genotype

Heterologous expression of hydrophobins RodA and RodB from Aspergillus fumigatus in host Pichia Pastoris

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Hydrophobins are small amphipatic proteins present on the spore surface of filamentous fungi. They express the hydrophobins RodA and RodB on the surface of its conidia and these may be of importance to the pathogenesis of the fungus. Although heterologous expression of hydrophobins has proven to be a challenge by past investigators, we made it the aim of this project to produce pure hydrophobins in sufficient quantities for further characterisation and investigation using the expression host Pichia pastoris.

Methods and materials: The genes encoding hydrophobins RodA and RodB were amplified by RT-PCR with gene-specific primers from the total RNA isolated from the spores of A. fumigatus (AF296 strain). The resulting cDNA was cloned into TOPO vectors using TOPO TA Cloning (Invitrogen), and the inserts were sequenced. The genes were further amplified by PCR to generate overhangs with specific restriction sites and cloned into expression vectors pPICZαA and pPICZB while adding a 6xHis-tag to the C-terminal of both hydrophobins. The pPICZαA vector expresses proteins with the signal sequence of alfa-mating factor from Saccharomyces cerevisia known to work well for protein secretion from P. pastoris strain X33 and transformants were selected by zeocin resistance. The presence of the RodA and RodB genes in the transformants was confirmed by colony PCR. The expression of RodA and RodB genes was induced by growing cells in culture flasks for three days in buffered complex methanol medium as protein production was dependent on the methanol-induced AOX1 promoter. The protein production was analyzed by SDS-PAGE, coomassie and silver-stained, as well as western blotting using a detection antibody (Penta-His HRP conjugate, Quagen). Recombinant RodA and RodB were purified using His-select Nickel Affinity gel (Sigma-Alrlich, Saint Louis, MO, USA).

Results: P. pastoris cultures expressing hydrophobins resulted in increased foaming, which was attributed to the presence of secreted hydrophobins. Protein bands of expected size were detected in both the fermentation broth and the foam by SDS-PAGE and western blotting. Optimization of the purification of hydrophobins and functional investigations are being carried out at the moment.

Conclusion: Hydrophobins RodA and RodB from Aspergillus fumigatus were successfully expressed and secreted by yeast host Pichia pastoris.

Keywords: Aspergillus fumigatus, Pichia pastoris, recombinant protein, hydrophobin, RodA, RodB, fungal spore.
Human pathogens, nosocomial infections, heat-sensitive textile implants and an innovative approach to deal with them

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Implantable polymers, as used for biomedical applications, inherently have to be sterile. Most implants however—particularly biomaterials as being developed in recent years for scaffold and tissue engineering—are heat sensitive. Therefore, the use of hazardous (radio-) chemicals is—due to the lack of alternative methods—still state-of-the-art for sterilisation processes. Furthermore, the aforementioned treatments often lead to the formation of persistent radicals that have been proven to cause allergic reactions, inflammations or even hemolysis when applied to human tissue [1]. Also, the use of chemical agents bear immediately dangers for natural resources and staff handling these chemicals [2] resulting in organisational and safety-related issues—especially with REACH regulations.

High-pressure CO2 treatment is a low-temperature technique that is already in use for pasteurisation of various liquid food products [3]. Using ANOVA and statistical modeling, it has been shown, that vegetative microorganisms adherent to solid surfaces can be inactivated using supercritical CO2 [4]. It exhibits mild treatment parameters, thus protecting sensitive textures; it speeds up reactions and mass transport due to the lack of phase interfaces. The CO2 pressure (process in adjoining figure) ranged from 50 to 100 bars and temperature was set to 25, 38 and 65 °C, investigating liquid, gaseous and supercritical state. Recent research has clearly shown that even pathogenic vegetative bacteria (C. albicans, S. aureus) can successfully be inactivated. The addition of small amounts (<< 1% w/w) of ozone finally assured inactivation of persistent spores (B. stearothermophilus, B. subtilis) of up to 10⁶ cfu/ml. Production of ozone and elimination after treatment takes places on-site; furthermore, CO2 can be recycled. Thus, the innovative sterilisation is virtually a zero-emission process unlike most conventional methods. It requires neither handling of hazardous chemicals nor quarantine storage of treated implants.

Typical implantable fabrics comprise of e.g. meshes and yarns made of biodegradable and non-biodegradable polymers. We investigated that the new treatment slightly decreased molecular weight and results in a reduced melting point of PP (polypolyene) and PLA (polylactic acid). PVDF (polyvinyliden difluoride) remains unchanged. Sensitivity surface measurement techniques- XPS, AFM, FTIR and contact angle show only minor oxidation and slight surface energy increase of polymers due to morphological and chemical effects. Further research found good biocompatibility of the treated fabrics by applying cell-toxicity (LAL) test as well as adherence test of L292 mouse fibroblast—observing enhanced growth on the treated surfaces. Positive results indicate that the eco-friendly treatment is a genuine alternative to conventional processes and could lead to further applications within the fields of medicine, drug processing and biotechnology.

**Keywords:** nosocomial infection, supercritical fluid, biopolymer, CO2 pressure


Identification of *Staphylococcus aureus* isolates from bovine mastitis samples related to human epidemic clone USA300 in backyard farms from México.

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**Staphylococcus* aureus*:** an innovative sterilisation process that causes diverse pathologies in humans and animals. Community-acquired (CA) strains cause infections in skin, upper respiratory tract, digestive tract (associated to alimentary intoxications) and toxic shock syndrome. Hospital-acquired (HA) strains cause pneumonia, endocarditis, osteomyelitis and sepsis, as well as quargulical-associated infections. Different *S. aureus* strains has been described as pathogens of skin infections in mammals, quargulical lesions in dogs and horses, and bovine mastitis. Strains sharing particular genetic backgrounds (Sequence Types and Clonal Complexes), as determined by Multilocus Sequence Typing (MLST), are describe for isolates of each of the pathologies described above. Clonal Complexes (CC) have been related to CA infections (CC8, CC30), HA infections (CC5, CC8) or bovine mastitis (CC97) among others. It is particularly interesting the recent widespread of the successful strain USA300 causing CA and HA infections in the USA. USA300 strain belongs to CC8 and has the Sequence Type (ST8) In this work we describe the isolation of USA300-related (ST8-related) isolates from bovine mastitis samples in two localities in the state of Michoacán, México. Backyard dairy milk farms are the most common production systems in this state and the most important economic activity for families with low incomes. Subclinical bovine mastitis is one of the most common health problems in these dairy herds. Molecular characterization of *S. aureus* isolates from bovine mastitis samples was done by MLST, macrorestriction analysis by pulse field gel electrophoresis (PFGE), polymorphism of proteina encoding gene (spa) and sequence of the 16S-23s ribosomal RNA gene spacer.

**Bovine mastitis epidemiology:** Nine farms were selected in Cotzio and Téjaro localities from the State of Michoacán. From 113 animals in lactation period 56 (49.6%) presented some degree of mastitis determined by the California Mastitis Test. From the total of quarters analyzed in positive animal were 30%, 20% and 4% for subclinical mastitis, while only 2.0% presented signs of clinical mastitis. Twenty one *S. aureus* isolates were obtained in *Staphylococcus*-110 agar and were positive to hemolisis and coagulase test. Identity of the isolates was confirmed by sequencing of the variable 3 region of the ribosomal RNA gene.

**Molecular caracterization of *S. aureus* isolates:** Twelve of the isolates were analyzed by MLST. Seven of them were ST97, two of them showed new alleles but were also related to CC97, an also related to CC97. Four of the reference strain (ATCC27543), isolate from bovine mastitis, showed also ST8. When analyzed by means of PFGE, the electrophoretic patterns were grouped by determining the Dice similarity coefficient. A similarity dendogram was constructed using an UPGMA algorithm from GeneDirectory application (Syngene). The dendogram showed that PFGE was able by itself to group isolates in ST97- and ST8-related branches. Size polymorphism analysis of spa gene in a larger collection of *S. aureus* isolates from the same region revealed the presence of at least seven different allelles. Six of these alleles were present in our isolates, but there was no evident correlation between ST or PFGE with the spa allele. Four isolates of the ST97, three isolates of the ST8 and the reference strain that also showed ST8, were used for analysis of the sequence of 16S-23S rRNA spacer. A region of approximately 80 bp was identified that is present exclusively either ST97 or ST8 isolates. Comparison of these sequences with rRNA databases revealed that our isolates of the ST8 and the reference strain shared homology with the sequence form the strain USA300-FPR375 a sucessful epidemic clone related to CA infections in the USA that is also methicillin-resistant. Two of our isolates showing ST8 were also resistant to low levels (2-4 μg/ml) of methicillin.

**Concluding remarks**, Bovine mastitis isolates bearing the ST8 have been also reported in Japan and The Netherlands. Since USA300 is a successful epidemic clone with an ST8 genetic background, it is needed to develop rapid identification methods for the precise detection of ST8-related isolates to strenght epidemiological surveilllace.

**Keywords:** *Staphylococcus aureus*, epidemiology, diagnostics
In vitro evaluation of biopolymers as delivery system of plant extracts on cultures of Porphyromonas gingivalis and Aggregatibacter actinomycetemcomitans

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Periodontal disease is an infectious and inflammatory process that affects the periodontium including the gingival, gingival attachment, periodontal ligament, cementum and supporting alveolar bone, and is the major cause of tooth loss in adults.

Therapy includes oral hygiene education, instrumentation for removal of calculus (scaling) and chemotherapy. A number of antibiotics administered systemically have been evaluated for periodontal therapy, such as tetracyclines, clindamycin, and metronidazole. These drugs have adverse effects like kidney and liver disorders, colitis, etc. As more antibiotics are employed, the development of resistance is an increased probability.

At recent years, the use of natural sources like plant extracts are enjoying great popularity. In Mexico, Thymus vulgaris, Croton lechleri and Julliana adstringens Schl. have been used to treating different infections.

The aim of this work was to use biotechnology in the development of a local delivery system for the treatment of periodontal disease evaluating antimicrobial properties of two natural polymers (Chitosan and Pullulan) added with aqueous extracts of T. vulgaris, C. lechleri and J. adstringens Schl. against Porphyromonas gingivalis and Aggregatibacter actinomycetemcomitans, the most frequent causative agents of periodontitis.

Chitosan and Pullulan are polysaccharide biopolymers that combine a unique set of versatile physicochemical and biological characteristics which allow for a wide range of applications, including biodegradability, biocompatibility, and nontoxicity.

Different parts of these plants were collected and extracted with solvents to obtain aqueous and organic extracts. The extracts were added to biopolymers in concentrations of about 10% to T. vulgaris, 11% J. adstringens Schl. and 20% C. lechleri. The polymers were used at 1% Chitosan and 10% Pullulan.

Values of the MIC were determined by a broth microdilution assay. Briefly, serial twofold dilutions of the antimicrobial agent were prepared in the appropriate culture medium in sterile 96-well roundbottom polystyrene microtiter plates. MBC were evaluated by broth disk test using disks of the polymers added with the three plant extracts.

The Pullulan biopolymers added with T. vulgaris showed zones of growth inhibition about 9mm of diameter, while J. adstringens Schl. showed 7mm for A. actinomycetemcomitans. Pullulan with J. vulgaris and C. lechleri had 6mm for P. gingivalis.

The Chitosan biopolymers with C. lechleri showed inhibition zones of 6mm for A. actinomycetemcomitans and 4.5mm on P. gingivalis. The biopolymers without extracts have inhibitory effects on Porphyromonas gingivalis and Aggregatibacter actinomycetemcomitans cultures.

Our findings suggest on the use of Chitosan and Pullulan showed synergistic activity with Thymus vulgaris, Croton lechleri and Julliana adstringens Schl., extracts for antimicrobial inhibition of Porphyromonas gingivalis and Aggregatibacter actinomycetemcomitans and have potential application as drug delivery systems.

Keywords: Thymus vulgaris, Croton lechleri, Julliana adstringens Schl., Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans antimicrobial activity, Chitosan, Pullulan.

In vitro tests of antimicrobial activity of plants used in Mexican traditional medicine

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In recent years fungal infections have been increasing due to a growing number of immunosuppressed and medically compromised patients. Candida is one of the most-common isolate in nosocomial bloodstream infections in the USA. Therapy includes oral hygiene education, instrumentation for removal of calculus (scaling) and chemotherapy. A number of antibiotics administered systemically have been evaluated for periodontal therapy, such as tetracyclines, clindamycin, and metronidazole. These drugs have adverse effects like kidney and liver disorders, colitis, etc. As these drugs have only fungistatic activity, justify the search for new strategies as the use of natural products.

Thymus vulgaris (Thyme), Croton lechleri (Sangre de Dragón), Julliana adstringens Schl. (Cuachalalate), Matricaria chamomilla (Manzanilla) and Caléndula officinalis L. (Caléndula) are known to have medicinal activity and some of the plants are used in Mexican traditional medicine to treat a wide range of infections. In the present study we investigated the antifungal activity of this five plant extracts.

The Chitosan biopolymers with C. lechleri showed inhibition zones of 6mm for A. actinomycetemcomitans, showed inhibition zones of 4.5mm on P. gingivalis. The biopolymers without extracts have inhibitory effects on Porphyromonas gingivalis and Aggregatibacter actinomycetemcomitans cultures.

Our findings suggest on the use of Chitosan and Pullulan showed synergistic activity with Thymus vulgaris, Croton lechleri and Julliana adstringens Schl., extracts for antimicrobial inhibition of Porphyromonas gingivalis and Aggregatibacter actinomycetemcomitans and have potential application as drug delivery systems.

Keywords: Thymus vulgaris, Croton lechleri, Julliana adstringens Schl., Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans antimicrobial activity, Chitosan, Pullulan.
**Indigenous medicinal plants popularly used for cure of bacterial diseases in Kishoreganj district of Bangladesh**

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Indigenous medicinal plants are widely used world wide to address a variety of human health problems. Alternative medicinal practitioners form an important component of the primary health care system of Bangladesh. Alternative medicinal practitioners generally possess considerably expertise on use of indigenous medicinal plants used to treat various human health ailments. The ailments treated can range from minor ailments like coughs and colds to difficult to treat diseases like cancer. Since the alternative medicinal practitioners in various regions of Bangladesh differ in their uses, a study was carried out to identify the potential sources for the development of new antibacterial drugs from indigenous medicinal plants found in Kishoreganj district of Bangladesh.

**Keywords**: Kishoreganj district, Meghna and Brahmaputra rivers, Alternative medicinal practitioners.

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**In vitro tests of polymers with extracts from plants used in Mexican traditional medicine on Candida albicans biofilm formation**

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The increase of fungal resistance to classical drugs, the treatment costs, and the fact that most available antifungal drugs only have fungicidal activity, justify the need for new strategies that make use of natural products and biotechnology. The aim is to find new effective antifungal agents from Thymus vulgaris, Croton lechleri and Juliana adstringens Schl. against Candida albicans using these extracts in Quitosan and Pullulan polymers. Different parts of these plants were collected and extracted with solvents to obtain aqueous and organic extracts. These extracts were added to biopolymers in concentrations about 10% to T. vulgaris, 11% J. adstringens Schl. and 20% C. lechleri. The polymers were in 1% Quitosan and 10% Pullulan. These polymers with extracts were tested in vitro for their antifungal activity against C. albicans biofilm formation. C. albicans is an opportunistic pathogenic fungus and is the most frequent causative agent of candidiasis. Candidiasis is an increasing health threat to immune compromised individuals and infections are commonly associated with the formation of biofilms, which are defined as complex microbial communities attached to a surface and encased in an exopolymeric matrix. Within this niche, C. albicans has intrinsic resistance to commonly used antifungal drugs. This study makes use of a 96-well microtiter plate model C. albicans biofilm formation to test the susceptibility of biofilms to the plant extracts. This model is coupled with a colorimetric 4XTT-reduction assay in which metabolically active sessile cells reduce a tetrazolium salt to water-soluble orange formazan compounds, and the intensity this product can be determined using a microtiter-plate reader.

Our results revealed that the Pullulan biopolymers of J. adstringens Schl. inhibited biofilm formation about 100%, while T. vulgaris showed low activity (2.5% biofilm inhibition), and C. lechleri inhibited biofilm formation about 52.5%. The Quitosan biopolymers showed activity with T. vulgaris and C. lechleri, with 51.5% and 44% biofilm inhibition, respectively. However, there was low activity using J. adstringens Quitosan biopolymer. Our finding showed that J. adstringens biopolymer exhibited strong antifungal activities in C. albicans biofilm inhibition in Pullulan, while T. vulgaris and C. lechleri exhibited inhibitory effects in Quitosan biopolymers. The biopolymers did not have any inhibitory effects on preformed C. albicans biofilms.

**Key words**: Thymus vulgaris, Croton lechleri, Juliana adstringens Schl., Candida albicans, antimicrobial activity, biofilm, Quitosan, Pullulan.
Influence of culture conditions on hydrogen peroxide production by *Lactobacillus jensenii*

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*Lactobacillus* is known to be the dominant bacterial genus harbored within the reproductive tract of healthy women and has been shown to protect women from vaginal infections through a variety of mechanisms. One mechanism that *Lactobacillus* uses involves the production of antimicrobial chemicals such as hydrogen peroxide to kill or inhibit pathogenic microorganisms. In spite of the health and ecological significance, little is known about how *Lactobacillus* makes hydrogen peroxide and the optimum conditions for its production. The purpose of this study was to determine how hydrogen peroxide production by *Lactobacillus jensenii* is influenced by *in-vitro* environmental factors such as glucose concentration, pH, and temperature, or by growth stage. *Lactobacillus jensenii* is a predominant species isolated from the human vagina. Hydrogen peroxide production from *Lactobacillus jensenii* was lowest at the highest glucose concentration (1% w/v) tested. This datum supports the hypothesis by Barnard and Stinson (1999) that an organism will conserve energy (reduce synthesis of antimicrobial chemicals) when carbohydrates are abundant. It is when carbohydrates are abundant that competition is less important. At 0% glucose, hydrogen peroxide production was higher than at the 1% glucose concentration, indicating that hydrogen peroxide may be effective against competition when carbon is limited. The highest rates of hydrogen peroxide production were observed at mid-range levels of glucose. It is possible that these levels of glucose supported a slower growth rate due to limited carbon availability, making the need to suppress competition crucial. In general, hydrogen peroxide production was higher for *Lactobacillus jensenii* at neutral pH and lower at acidic pH levels. The higher production of hydrogen peroxide at neutral pH may be an antibacterial response to competition from other microorganisms which occurs during an infection such as bacterial vaginosis. Bacterial vaginosis often results in an increased vaginal pH. The lower hydrogen peroxide production observed at acidic pH levels may represent a mechanism *Lactobacillus* uses to conserve energy, because the acidic environment itself would inhibit the growth of pathogenic organisms. A significantly lower rate of production occurred at 30°C, the lowest temperature tested, than at all other temperatures. The peak rates of production occurred at the two highest temperatures tested (40°C and 45°C), indicating that increased temperature enhances the production of H2O2 in *Lactobacillus jensenii*. The relationship of hydrogen peroxide production and growth of *Lactobacillus jensenii* was reported two different ways. When calculated as hydrogen peroxide/minute, it was observed that high cell numbers resulted in high hydrogen peroxide production. When calculated as hydrogen peroxide/minute/OD unit, production coordinated with growth stage rather than cell numbers. *Lactobacillus jensenii* produced higher hydrogen peroxide during early log growth when nutrients are in excess and when cells are focused on primary metabolism. In conclusion, this research indicates that many factors influence hydrogen peroxide production by *Lactobacillus jensenii*.

**Keywords** *Lactobacillus*, hydrogen peroxide

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Influence of patients' care with invasive devices on the risk of health care-associated infections

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The risk of health care-associated infection are associated with being in the intensive care unit (ICU), undergoing surgery, and invasive procedures. Although not all catheter-associated urinary tract infections and bloodstream infections can be prevented, it is believed that a large number could be avoided by the proper management of patients' care with invasive devices. The purpose of the study was to investigate the influence of patients' care with invasive devices (urinary catheters and peripheral venous catheters) on the risk of health care-associated infections. Quantitative and qualitative research methods such as questionnaire, clinically structured empirical research and microbiological tests was used in the ICUs and in the surgical units of multi-profile Hospitals of Latvia. By means of microbiological investigation the contamination level of the ICUs and surgical units was determined: a) using a Count-Tact applicator and a culture medium specially selected for this method, b) using the swab method for the microbiological contamination of the equipment and nurses' hands, and c) using the catheter sedimentation method for the microbial contamination of the invasive devices. In total 166 microbiological samples and 45 observation objects was analysed. Results suggested that a common problem in ICUs and in surgical units is the lack of unified nursing protocols on performing invasive procedures. There was excessive workload of nurses working in ICUs and in surgical units. Results showed disregard for basic principles of hand hygiene and aseptic mistakes during patients care with invasive devices. Microbiological investigations showed a high level of bio-contamination on the nurses' hands during invasive procedures and medium to high levels of bio-contamination were discovered on patient's changed bed sheets as well as in nurses' hair and their workwear. Mentioned factors influence the patients care with invasive devices and induce risk for HAIs.

**Keywords** invasive device, health care-associated infection

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Inhibition of Endothelial Interleukin-8 Production and Neutrophil Transmigration by Staphylococcus aureus Beta-Hemolysin

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Neutrophils play a crucial role in the host response to infection with Staphylococcus aureus, which is a major human pathogen capable of causing life-threatening disease. Interleukin-8 (IL-8) is a potent chemoattractant and activator of neutrophils. We previously reported that S. aureus secretes a factor that suppresses IL-8 production by human endothelial cells. Here we isolated an inhibitor of IL-8 production from the supernatant and identified it as staphylococcal beta-hemolysin. Beta-hemolysin reduced IL-8 production without cytotoxicity to endothelial cells. Pretreatment with beta-hemolysin decreased the expression of both IL-8 mRNA and protein induced by tumor necrosis factor alpha (TNF-alpha). Migration of neutrophils across TNF-alpha-activated endothelium was also inhibited by beta-hemolysin. In contrast, beta-hemolysin had no effect on intercellular adhesive molecule 1 expression in activated endothelial cells. These results showed that beta-hemolysin produced by S. aureus interferes with inflammatory signaling in endothelial cells and may help S. aureus evade the host immune response.

Keywords: Staphylococcus aureus; IL-8; endothelial cell

Invasive infection by Trichosporon mucoides following circovirus infection in a parrot

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Cryptococcus neoformans, Histoplasma capsulatum, Coccidioides immitis, Blastomyces dermatitidis, Pneumocystis carinii, Aspergillus spp, Candida spp, and other less common fungi have been identified as causative agents of mycotic pneumonia in animals. These agents are often found in immunocompromised hosts, but can cause disease in healthy individuals as well. Pulmonary tissues and secretions constitute an excellent environment for the growth and development of these organisms. The soil is their primary source, being the main reservoir of those agents, which can be acquired by inhalation or skin abrasion.

Trichosporon is a natural inhabitant of the soil but can, occasionally, be found as a component of the normal skin mycobiota of animals. It is also found as a saprophytic coloniser of some mucosae like the throat and lower gastrointestinal tract. Although uncommon, Trichosporon has been recently recognised as an opportunistic pathogen that can elicit a potentially fatal systemic infection in immunocompromised hosts. Several cases of disseminated T. mucoides infection have been reported recently but in Portugal no necropsy data or report are available.

As far as we know, this report describes the first fatal case of disseminated trichosporosis caused by T. mucoides in a 6 months old immunocompromised parrot. The animal also revealed to hold an infection due to Circovirus. Avian circovirus infections can cause different clinical manifestations but in all cases a lymphoid depletion is observed, associated to an immunosuppression that favours the appearance of opportunistic secondary infections. Histopathological lesions were suggestive of severe pulmonary infection with intense hyphae proliferation which accumulated in the parabronchi with simultaneous invasion of the blood vessels. T. mucoides was grown on Sabouraud agar with chloramphenicol (BD Diagnostics-254091) and the identification of this fungal species was done both by morphological characterization and biochemical tests (ID 32 C - Biomerieux 32 200). Circovirus was detected by PCR and the amplified fragment was confirmed by sequencing. The parrot tested negative for avian influenza by RT-PCR and for Pacheco’s disease virus by PCR.

Keywords: Systemic Mycotic infection, Trichosporon mucoides, parrot, circovirus
Investigation of in-vitro and in-vivo Beta-Lactamase Inhibition by Beta-Lactamase-Inhibitor-Protein Based Peptides

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Beta-lactamase is an important drug target in combating the increasing problem of beta-lactam antibiotic resistance. To overcome beta-lactamase mediated antibiotic resistance towards beta lactam antibiotics, inhibitors such as clavulanic acid, sulbactam and tazobactam are commonly used in conjunction with various beta-lactam antibiotics. Streptomyces clavuligerus beta-lactamase-inhibitor-protein (BLIP) has been shown to be a potent inhibitor of class A beta-lactamases including the Escherichia coli TEM-1 beta-lactamase. Therefore, beta-lactamase inhibition by BLIP is an interesting research field for peptide based inhibitor development. In the current study, E. coli K12 strain harboring the pUC18 plasmid that carries the gene of RTEM-1 beta-lactamase, was used for periplasmic beta-lactamase production. To investigate in-vitro and in-vivo TEM-1 beta-lactamase inhibition, BLIP based peptides of different lengths were tested. In-vitro beta lactamase inhibition was observed by both peptides at a concentration of 400 μM. This interaction was verified by affinity SDS-page. Based on the fact that biotinylated peptides can readily be transported into gram (-) bacteria such as E. coli, in-vivo beta-lactamase inhibition was also investigated by the same BLIP derived peptides with N-terminal biotinylation.

Keywords: antibiotic resistance, BLIP, peptide inhibitor, beta lactamase inhibition

Isolation and Identification of ATP-secreting bacteria from mice and humans

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In a recent report, ATP was shown to cause colitis in mice via Th17-cell differentiation (Atarashi K, et al. Nature 2008). Although it was suggested that ATP was secreted by commensal bacteria in the murine intestine, the ATP-secreting bacteria have not been isolated and identified. In the present study, we have isolated and identified the ATP-secreting bacteria from mice and humans. In humans, assessment of the relationships between the ATP-secreting bacteria and colitis will facilitate the understanding of various aspects of human colitis, including the pathology, development of treatment avenues, prophylaxis, and prognosis. Since ATP is essential for the organisms, the extracellular secretion of ATP may indicate unknown symbiotic relationships with some pathogen or commensal in the microbial flora of the gut.

Keywords: ATP-secreting bacteria, colitis, commensal, pathogen, microbial flora.
Isolation and screening of soil microorganisms for membrane-active antimicrobial metabolites

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Resistence of microorganisms to antibiotics has become a major problem in the treatment of infectious disease. Many of the surviving microbial pathogens are resistant to common antibiotics, and there is an ongoing search for new anti-infective agents. Many traditional antibiotics are produced naturally by soil microorganisms for the purpose of competing microbial species in their biologically diverse environment. Moreover, microorganisms are constantly changing, and adapting to new situations. In addition, over the past 30 years a number of new infections have been discovered. With the continuation of this process, it is important to continue to find anti-infective agents with fewer side effects, shorter lengths of treatment and in particular, drugs with new and less resistant targets to antimicrobial activity. Although antibiotics have various modes of action to inhibit or stop the growth of microorganisms, like inhibition of cell wall formation, protein, nucleic and ribonucleic acid synthesis etc. membrane as a new and potential target is noteworthy for antibiotics action because of less tendency to develop resistance.

In this study soil samples were collected from desert and farming zones of Iran. 3 selective media were used for cultivation of soil dilutions to grow bacterial, fungal, and actinomycete colonies on agar surface. A total number of 75 species consist of 36 bacteria, 28 fungi and 11 actinomycetes were isolated from the soil samples. In the primary screening that was performed to evaluate antimicrobial activity, isolated microorganisms were analyzed using the overlay agar technique in terms of their general inhibition effects to indicator strains *E. coli*, *C. albicans*, and *S. cervisiae*. It has been found that 23 isolates including 4 bacteria, 13 fungi and 6 actinomycetes, were effective against test microorganisms. In the secondary screening to determine membrane-active metabolites producing microorganisms, isolates which had an inhibitory effect against test microorganisms, were analyzed for membrane activity using a Rapid Chromatic Detection method by use of a Biomimetic Polymer Sensor in conjunction with phospholipid as a membrane model. Based on color and fluorescence changes that are easily identified by the naked eye and fluorescent microscope, 3 species consist of 1 fungus and 2 actinomycete had membrane-activity effect and were stored for the sake of further study and identification.

**Keywords** microorganism; soil; antimicrobial activity; membrane; Persian Gulf

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Isolation, identification and antimicrobial activity of an *Aneurinibacillus* strain from bat cave of tropical rain forests in Thailand

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A gram positive sopre froming strain was isolated, in the course of the routine screening of bacteria for industrial purposes, from the bat cave of tropical rain forests in Southern Thailand. Colony morphology, biochemical tests and chemotaxonomic investigations revealed that this strain had the characteristics of the family *Paenibacillaceae*. Comparative 16S rRNA gene sequence analysis showed that the organism was the most closely related to *Aneurinibacillus migulanus* with 98% similarity. This strain was inhibited by strains of *Bacillus anthracis*, methicillin resistant *Staphylococcus aureus* (MRSA), and vancomycin-resistant Enterococci (VRE). These findings suggest that this bacterial strain might have a potential as an antimicrobial agent, especially against superbacteria.

**Keywords** *Aneurinibacillus migulanus*; antimicrobial agent; superbacteria
Lactic acid bacteria from the vagina of healthy Turkish women: identification, hydrogen peroxide production

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Lactic acid bacteria are ubiquitous in nature and in humans they play a very significant role in the general health maintenance of the host.

Lactic acid production is considered to be the major protection mechanism of lactobacilli against vaginal infections due to genital pathogens. Some species of Lactobacillus are also hydrogen peroxide (H$_2$O$_2$) producers.

The present study was conducted to identify the species of lactic acid bacteria isolated from vaginal fluids of reproductive-age women and to characterize the H$_2$O$_2$-producing an healthy Turkish women.

Identification of lactic acid bacteria have previously been based on culture-dependent methods and ribotyping. Ribotyping was performed with the RiboPrinter Microbial Characterization System (Qualicon Inc., Wilmington, DE) and the standard EcoRI DNA preparation kit, as described in the manufacturer's operations and analytical guides.

We identified Lactobacillus paracasei spp. paracasei, L. brevis, L. delbrueckii subsp. Delbrueckii, L. plantarum, Lactococcus lactis, Leuconostoc mesenteroides, and as the most frequent species. In this healthy Turkish women had H$_2$O$_2$-producing vaginal lactobacilli.

Keywords: Lactic acid bacteria, Vagina, H$_2$O$_2$.

Methicillin-resistant Staphylococcus aureus (MRSA) in Brazil: Classification of SCCmec and virulence factors

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Staphylococcus aureus strains are responsible for a wide variety of clinical manifestations, which generally depend on the numerous virulence factors produced by each strain. These factors include adhesion molecules, biofilm formation, and the production of enterotoxins, exfoliative toxins and leukocidins. The objective of the present study was to detect methicillin-resistant S. aureus (MRSA) in clinical and/or surveillance cultures obtained from patients seen at a teaching hospital, and to associate the findings with the prevalence of resistance and virulence factors. A total of 424 S. aureus strains were isolated and identified in surveillance cultures, burn swabs, blood cultures, secretions and other clinical specimens obtained from 123 patients hospitalized at Hospital Estadual Bauru (HEB), Faculdade de Medicina de Botucatu (FMB), UNESP, Brazil. Next, the resistance profile of these strains was determined by the agar disk diffusion technique using the following antibiotics: oxacillin (1 µg), cefoxitin (30 µg), erythromycin (15 µg), gentamicin (10 µg), and vancomycin (30 µg). Genotyping of these strains was only performed for the first positive sample of each patient (blood culture or other clinical material, if available). According to these criteria, the genotypic resistance and virulence profiles of 212 S. aureus strains were analyzed. PCR for detection of the mecA gene was used for genotypic analysis of resistance and positive strains were submitted to subtyping of the staphylococcal cassette chromosome (SCCmec) by multiplex PCR. For genotypic analysis of virulence factors, PCR was used for the detection of the following genes: enterotoxins A, B and C (sea, seb and sec-I), toxic shock syndrome toxin ( tst ), Panton-Valentine leukocidin ( LukPV ), alpha ( hla ) and delta hemolysins ( hld ), exfoliative toxins A, B and D ( eta, etb and etd ), and biofilm formation ( icaA and icaD ). Investigation of the mecA gene revealed 134 (63.2%) MRSA strains. Characterization of SCCmec in these strains showed the presence of SCCmec type III or variations of type III in 104 (77.6%) strains, SCCmec type I or IA in 23 (17.2%), SCCmec type IV in 4 (3%), and SCCmec type II in 3 (2.2%). Analysis of the virulence profile showed no significant difference in the presence of genes encoding biofilm production ( icaA and/or icaD ), delta hemolysin ( hld ), alpha hemolysin ( hla ), or enterotoxin in A ( sea ) or B ( seb ) between MRSA and MSSA. However, the enterotoxin C gene ( sec-I ) was more frequent in MRSA strains (42.5% versus 20.5% in MSSA). The importance of these pathogens is related to the combination of virulence mediated by their toxins, their invasive character and resistance to antibiotics. Thus, determination of these factors permits the establishment of the resistance and virulence profile of these circulating pathogens and, consequently, to maximize measures for the control, treatment and prevention of these microorganisms.

Keywords: Staphylococcus aureus, MRSA, SCCmec, Antimicrobial Agents, Biofilm, Toxins, Hemolysins, Virulence Factors.
Microorganisms of the peloid: *Pseudomonas sp.* and *Arthrobacter sp.* as probable producers of prostaglandins

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It is shown, that *Pseudomonas sp.* and *Arthrobacter sp.*, found in the peloid of health resort Garden-town(near Vladivostok), synthesize de novo from 10 up to 14 fatty acids, respectively, as methyl esters and only the small part is presented by free fatty acids.

It is shown also both *Pseudomonas sp.* and *Arthrobacter sp.* produce the same type of prostaglandins also as a methyl ester, methyl ester of prostaglandin of group E.

Keywords fatty acids, peloid, prostaglandins

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New antibacterial molecules produced by endophytic *Paenibacillus polymyxa*

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Endophytic bacteria can produce complex natural products and as such are recognized as a source of new antibacterial agents with potentially significant impact on treatment of infections. A strain of Gram-positive aerobic endophytic microorganism was isolated from the leaves of *Prunus* spp., a Brazilian tropical savannah tree. The 16S RNA analysis indicated that the isolate was *Paenibacillus polymyxa*. This isolate, designated *P. polymyxa* RNC-D, was evaluated for its antimicrobial activity against important pathogenic bacteria. For production of soluble activity, the microorganism was cultivated in ISP2 broth at 28°C with agitation. When stationary phase was attained (48-120h) maximum antimicrobial activity was observed in the cell-free supernatant. Quantitative evaluation of the crude soluble activity was done using a well diffusion assay in which two-fold serial dilutions of the extract were tested against *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 as test strains. The stability of the antimicrobial activity was evaluated. A 10-fold crude extract concentrate was obtained using a speed-vac and then tested for enzymatic, thermal and pH susceptibility. The bioactivity was resistant to proteases (pronase, pepsin, trypsin, protease K) but was sensitive to lipase. It was resistant to thermal treatment up to 121°C for 15 min, could be frozen (minus 80°C) and was stable at pH varying from 2.0 to 9.0.

Filtration on a Ultrafree (Millipore) membrane with cut-off of 2,000Da showed that the antimicrobial activity was of low molecular weight as it was found in the filtrate only. Using anionic chromatography on a Mono-Q column coupled to an Akta purifier system (GE Healthcare) we could resolve peaks showing variable activity. From these peaks, using Mass spectroscopy, we could conclude that some represented compound already known to be produced by *P. polymyxa*, like polymixin E and F04 peptide. Nevertheless, two peaks corresponded to new molecules with antimicrobial activity that can represent new therapeutic tools. One of these substances is a tripeptide (Glu-Cys-Gly) and is active against *E. coli*. The other molecule is lipidic in nature and is active against *S. aureus*. The chemical nature of both newly identify antimicrobial molecules explain the chemical stability observed in the well diffusion assay.

Keywords: Antimicrobial activity, endophytic bacteria, biochemical characterization, purification, *Paenibacillus polymyxa*. 
Phylotaxonomy of human and environmental isolates belonging to *Tissierella* group.

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Bacteria belonging to the *Tissierella* group, which includes the genera *Tissierella*, *Tepidimicrobium*, *Soehngenia* and *Sporanaerobacter*, are anaerobic gram-positive bacilli currently classified as *Peptostreptococcaceae* in the phylum *Firmicutes*. So, their phylotaxonomy remains unclear. These bacilli were described from environmental sources such as activated sludge and hot springs. *Tissierella praecuata* has been also reported as an opportunistic human pathogen. We used a polyphasic approach in order to clarify the phylogeny and the taxonomy of the *Tissierella* group as well as to describe the diversity of its members. The 16S rDNA based phylogeny was reconstructed on 453 sequences. Electron microscopy, multi-locus sequencing (tpi, recA and spo0A genes) and determination of the genomic skeleton by pulse-field gel electrophoresis were performed on 27 clinical isolates and reference strains. We confirmed the phylogenetic placement of the *Tissierella* group in the phylum *Firmicutes* as well as their gram-positive cell wall structure, in spite of their negative reaction to gram stain. In the phylogenetic tree, the *incertae sedis* families XI, XII and XIII, the family *Peptostreptococcaceae sensu stricto* and several members of the family *Clostridaceae* were gathered in a robust clade that branched at the same depth than previously defined classes in *Firmicutes*. Therefore, we proposed to affiliate the clade to *Peptostreptococcaceae* class nov. within the *Firmicutes* phylum. Associated to gram-positive cocci (*Anaerococcus, Finegoldia, Gallicella, Prevotella, Parvimonas, Peptoniphilus*), the *Tissierella* group formed a new *order* (or family rank) within this class. Among the clinical isolates firstly affiliated to *T. praecuata*, two distinct bacterial species were distinguished on the basis of 16S rRNA, tpi, recA and spo0A gene sequencing: *T. praecuata* and a novel one (5 isolates). Each species displayed specific characteristics with regard to genomic size and chromosomal skeleton structure. Deposited 16S rDNA sequences probably affiliated to the new species have been identified by others in samples from various sources (ramen or yak, digestive tract of termite or digester of biogas plant). The non-described *Tissierella* species represents a new human opportunistic pathogen that may be involved in several human infections such as bacteremic and osteo-articular infections. The epidemiology of such opportunistic pathogens as well as their reservoir are worthy of further investigation.

**Keywords:** *Tissierella*, phylogeny, environment, opportunistic pathogens

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**Pneumococcal whole-cell vaccine: optimization of pneumococcal cell growth using fed-batch cultivation**


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The current vaccines against *Streptococcus pneumoniae* are composed of capsular polysaccharides from serotypes which are prevalent in USA and Europe. The free polysaccharide vaccines are effective only in adults and conjugate vaccines are effective in children, but their high cost has not allowed their inclusion in mass campaigns in developing countries. Besides, there are cases of serotype replacement reported in the countries that have already included conjugate vaccines in the schedule for children vaccination. In order to solve these problems, Malley et al. developed a pneumococcal whole-cell vaccine, which is based on an unencapsulated strain of *S. pneumoniae* and therefore it is a serotype-independent vaccine. Moreover, its production process is simpler and more inexpensive than the other pneumococcal vaccines, since it is unnecessary to purify the antigens which are present in the vaccine. *S. pneumoniae* is a fastidious anaerobe aerotolerant microorganism; its metabolism is restricted to substrate level phosphorylation and its main product is lactic acid, which imposes strong constraints to the biomass yield due to both: unfavorable energy balance and end-product inhibition of growth. The present work aimed to increase the pneumococcal cell growth using different fed-batch conditions and to define the most economically advantageous feeding medium.

Strains Rx1Al kan<sup>R</sup> and Rx1Al P1 kan<sup>R</sup> were used in this work, the strain P1 is deficient in pneumolysin activity and both strains are unencapsulated strains with an insertion duplication mutation in the *lytA* gene of the major pneumococcal autolysin. Two batch media were used containing either 5 or 20 g/L mecanically hydrolyzed soybean meal (EHS). The other components of batch media were 20 g/L yeast extract (YE), 0.01 g/L sodium lactate, 0.1mM CaCl<sub>2</sub>, 0.625 g/L glutamic acid, 5 m/L MgSO<sub>4</sub>, 0.8 g/L Na<sub>2</sub>SO<sub>4</sub>, 0.36 g/mL MnSO<sub>4</sub>, 200 mg/L kanamycin. Frozen stock culture (100μl) was used to inoculate 500 mL of the medium and incubated at 36°C and 3% CO<sub>2</sub> for 11 h. This pre-culture was inoculated into a bioreactor to obtain an initial OD of 0.1. Batch and fed-batch cultures were carried out in 10L-bioreactors (BioFlo2000, New Brunswick, USA) at 36°C, 150 rpm, 0.5 L/min, 0.1 bar, and pH control at 7.0 by addition of 5m NaOH. Polypropylene glycol was used as an antifoam agent. The fed-batch conditions were simulated using the software AmBio 1.2 with the Levenspiel’s end-product (lactate) inhibition model. Three feeding media were tested, each one presenting concentrated amounts of EHS, YE and glucose, and all other components at the same concentration than the batch medium: 1) 20 g/L EHS, 80 g/L YE and 80 g/L glucose; 2) 20 g/L EHS, 80 g/L YE and 80 g/L glucose; 3) 200 g/L EHS, 200 g/L YE and 200 g/L glucose. The cell growth was measured by OD at 600nm (Hitachi U1800 spectrophotometer). After centrifugation of culture broth samples at 20,000 g and 4°C for 10 min, glucose, lactate and acetate were determined in the supernatant using high-performance liquid chromatography (HPLC, Shimadzu using an Amiex HPX 87H column (300 x 7.8 mm, BioRad), at 60°C and 5 mM H<sub>2</sub>SO<sub>4</sub> solution was used as mobile phase with a flow rate of 0.6 mL/min.

For preparation of the whole-cell vaccine, the harvesting should be performed before the stationary phase, thus it should be done at OD~5.0 using the 5 g/L EHS medium and at OD~6.5 using 20 g/L EHS medium in batch cultures. After adjusting the Levenspiel’s model to the batch data, different fed-batch conditions were simulated and the promising ones were tested: a) batch phase with 5 g/L EHS medium followed by feeding medium “a”; b) the same as “a”, but feeding medium “b” at 0.4 L/h; c) batch phase with 20 g/L EHS medium followed by feeding medium “c” at 0.2 L/h; d) the same as “c”, but feeding medium “d” at 0.7 L/h. The OD of harvesting increased in all tested fed-batch conditions, reaching 9.0 for condition “a”, 9.5 for “b”, 10.0 for “c” and 11.5 for “d”. The present work applied simple tools of modeling and simulation to define fed-batch conditions and to achieve an important increase in pneumococcal biomass yield: from 2.25 g/L dry cell weight in batch to 4.27 g/L in fed-batch using the 5 g/L EHS medium, and from 2.92 g/L in batch to 5.17 g/L using 20 g/L EHS medium, i.e., the biomass production was almost two fold higher in fed-batch than in simple batch cultivation. Further studies should evaluate the impact of fed-batch production on the immunogenicity of the vaccine.

**Support:** PATH and FAPESP

**Keywords:** *Streptococcus pneumoniae*, fed-batch cultivation

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Polyphasic characterization of \textit{Aspergillus fumigatus} strains causing infection in parrots and dolphins

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\textit{Aspergillus fumigatus} is a common and ubiquitous mould and an opportunistic pathogenic fungus, being its spores an important component of the normal airborne microflora. Epidemiological data emphasize that \textit{A. fumigatus} can infect most animals, including humans, especially when the individuals are immunocompromised. The first case of human allergic bronchopulmonary disease due to this fungus was described in the mid-twentieth century, in London, and the first invasive and fatal infection in an immunocompromised patient was also described a few years later. Immune dysfunction allows the germination of inhaled spores and the growth of mycelia in animal and human lungs, causing an invasive condition known as aspergillosis.

The aim of this work was to characterize several \textit{Aspergillus fumigatus} strains isolated from eleven ornamental birds (parrots - \textit{Amazona aestiva}) and six dolphins (\textit{Delphinus delphis}) presenting culture-confirmed infection by this fungus. All dolphins presented also clinical signs of respiratory diseases which were refractory to anticoagulant treatment. A polyphasic approach was followed for the characterization of isolates, including the use of conventional phenotypic tests and molecular typing tools.

For strain isolation, swabs were taken from air sacs/lungs during necropsies of parrots and from nasal/pulmonary exudates of dolphins and transported immediately to the laboratory. Cultures were grown on two conventional media, Sabouraud Agar containing Gentamycin and Chloramphenicol and Cooke Rose Hengal Agar, incubated for five days at 25 °C as well as at 37 °C. Suspected \textit{Aspergillus} spp. colonies were purified and identified based on their respective macro and micromorphological features. Additionally, all strains were typed by a PCR-based assay involving the amplification of polymorphic regions of extracted genomic DNA using the microsatellite primer (GACA)₈.

In Portugal there are few published reports describing the morbidity and mortality of the infection caused by \textit{Aspergillus} spp. in animals. The present study documents the occurrence of natural and fatal infections in the respiratory tract of parrots and dolphins due to \textit{A. fumigatus} and the preliminary characterization of these agents.

\textbf{Keywords}: \textit{Aspergillus fumigatus}, Parrots, Dolphins, Polyphasic strain characterization

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Propolis from Azores: antimicrobial and antioxidant properties

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Propolis, or bee glue, is a natural mixture processed by honeybees (\textit{Apis mellifera}) from substances exuded by plants: lipophilic materials on leaves and leaf buds, resins, mucilages, gums and lattices. Apart from plant exudates, \textit{Aspergillus fumigatus} is an important component of the normal airborne microflora. Epidemiological data emphasize that \textit{A. fumigatus} is able to infect animals and cause disease.

\textit{A. fumigatus} is an important pathogen, being often the first invasive and fatal infection in an immunocompromised patient. Several cases of aspergillosis have been reported in parrots and dolphins. The present study documents the occurrence of natural and fatal infections in the respiratory tract of parrots and dolphins due to \textit{A. fumigatus} and the preliminary characterization of these agents.

\textbf{Keywords}: \textit{Aspergillus fumigatus}, Parrots, Dolphins, Polyphasic strain characterization
Proteinic extracts from *Sporothrix schenckii* cell wall a novel serological strategy by sporotrichosis

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*Sporotrichosis* is an opportunistic fungal infection that has become a global clinical importance in recent times due to the problems of HIV, cancer, diabetes and immunosuppression. This infection is caused by the dimorphic fungus *S. schenckii*. This disease is characterized by clinical lesion in the skin, whereas in the immunocompromised patient may spread systemically causing death. In clinical practice the diagnosis of sporotrichosis is complicated and in most cases the medical staff underdiagnosis the disease. Its diagnosis is based on the cultivation of the infected tissue biopsies. The serological methods used today in many false positives and negatives. The purpose of this study was to establish strategies through cellular, biochemical and immunologic methods to diagnose sporotrichosis. By null-LHLC LC Biotin was to mark the achievement of cell wall proteins of *S. schenckii*. The labeled proteins were used for the design of a ligand like assay. By incubating these proteins with epithelial tissue, it was observed that at least five proteins had affinity for this tissue (Mr > 190, 180, 115, 90 and 80 kDa). Those proteins were used as antigens immunization protocols classic rabbit immune serum was obtained with a 1:2500 title which reacts with the proteins of *S. schenckii* showed that the affinity for the epithelium. Western blot and indirect immunofluorescence assays showed that the serum immune present reaction toward proteinic extracts and different morphological stages ofclinical isolated strains of *S. schenckii*. Also for these tests showed that the immune serum generated no cross-reactivity with metabolic fractions of other non-pathogenic and pathogenic fungi. In sum, these results indicate that the immune serum could be useful for detecting *S. schenckii* in biological samples, but more experiments will be conducted to assess the biological effectiveness and employment as strategies basic in immunotherapy and diagnosis for this disease. DAIP -UG Grant No. 2008 to MSL.

**Keywords** Sporotrichosis; serological diagnosis

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**Purification of Cephamycin C from fermentation broth**

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Cephamycin C (CepC) is an important β-lactam compound, belonging to the class of cephalosporins. As a β-lactam antibiotic, it affects the peptidoglycan’s synthesis in prokaritotes, affecting the cell wall integrity. CepC is produced by the bacteria *Streptomyes clavuligerus* and *Nocardia lactamidurans* in submerged fermentations, and it has to be isolated from the fermentation broth using appropriate downstream techniques, in order to obtain the antibiotic at an adequate purity. In this work, it was investigated the purification of CepC applying the processes of ultrafiltration, nonspecific adsorption and ion-exchange chromatography. All assays utilized microfiltered culture broth from batch fermentations of *S. clavuligerus*. CepC was determined by bioassay, using the bacterium *Escherichia coli* ESS, and by HPLC. In the ultrafiltration studies, nine experiments were conducted to evaluate the influence of temperature (10, 15 and 20°C) and pressure (0,75, 1,00 and 1,25 Kgf.cm2). After ultrafiltration, the broth was clarified at an agitated tank by the adsorption of contaminants in the resin Amberlite XAD-4 (relation 3:1, broth:resin). The ion-exchange chromatography was carried out in a column packed with the resin Q Sepharose XL, 20°C, and the elution was made with NaCl solutions. Breakthrough curves with this ion-exchange resin were obtained to determine the best flow rate for the broth feed (2,5; 5 and 7,5 mL.h-1). Ultrafiltration experiments showed that flow rates of permeate, in all the cases, fell with filtration time. This fact indicated that the cake formation on the membrane increased the system resistance (membrane+cake). The average value of Rm was of 1.25x1014±9.23x1013. CepC concentration was practically the same in the permeate and concentrate fraction and the initial broth. In relation to contaminants, highest values were obtained in the concentrate fraction. RMN-1H analysis of the ultrafiltered broth and after treatment with the resin XAD-4 showed that the spectrum of the latter was cleaner than the one of the former. From this results, it’s possible to conclude that part of the contaminants were adsorbed by the resin, and, therefore, their hydrogen signals weren’t detected in the other spectrum. In the ion-exchange chromatography, a elution with NaCl solutions was able to separate two different fractions with antibacterial activity, detected by bioassay. The first peak was eluted passing 0,1% NaCl solution through the column, and the second was eluted with a solution of 0,5% NaCl. Only the fractions from the first peak could be detected by the HPLC method utilized. These results showed that ultrafiltration and adsorption with XAD-4 resin were able to clarify the broth, eliminating contaminants, and the ion-chromatography with QXL resin and gradient elution with NaCl solutions could separate different fractions from the broth.

**Keywords** cephamycin C; purification; ultrafiltration; ion-exchange chromatography; non-specific adsorption
Purinergic agents of hydroalcoholic *Agaricus blazei* extracts: identification, quantification and biological assays

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Agaricus blazei is popularly known as *cogumelo do sol* in Brazil. It is widely used today as an edible mushroom and as a natural therapy in the form of a medicinal extract against a variety of diseases like diabetes, atherosclerosis, hepatitis, hypercholesterolaemia, heart disease and others. Edible mushrooms contain many bioactive compounds, such as terpenoids, steroids, phenols, proteins and polysaccharides. More recently, nucleotides, nucleosides and nucleobases have also been isolated from several mushrooms, as for example, *Ganoderma lucidum* and *Lentinus edodes*. Several of them are present at relatively high concentrations, raising the suspicion that their functions go beyond the simple role of metabolic intermediates. In mammals this group of compounds is involved in the regulation and modulation of various physiological processes mediated by purinergic receptors, so that they could be responsible for some of the physiologic effects normally attributed to edible mushrooms. With respect to its nucleotide, nucleoside and nucleobase contents *Agaricus blazei* has not yet been investigated. Identification of this class of compounds in *Agaricus blazei*, however, could be useful to the attempts of explaining the physiologic and pharmacologic effects of this species. Taking this into account the present work had two main purposes: (1) to identify and quantify the nucleotides, nucleosides and nucleobases of *Agaricus blazei* hydroalcoholic extracts; (2) to test the extract for a possible purinergic action due to the presence of those compounds. Identification and quantification were done by means of high-performance liquid chromatography (HPLC). Samples of the fruiting bodies (basidiocarps) of *Agaricus blazei* were extracted with ethanol 70% and the extracts were freeze-dried and dissolved in water for analysis. The HPLC system (Shimadzu, Japan) consisted of a SCL-10AVP system controller, two model LC10ADVP pumps, a model CTO-10AVP column oven and a model SPD-10AVP UV-VIS detector. A reversed-phase C18 HRC-ODS column (5 μm; 150x6 mm ID; Shimadzu, Japan) protected with a GHRC-ODS pre-column (5 μm; 10x4 mm ID, Shimadzu, Japan) was used with a gradient from reversed-phase 0.044 mol/L phosphate buffer solution, pH 6.0, to 0.044 mol/L phosphate buffer solution plus methanol (1:1), pH 7.0, at 0.8 mL/min. The identification of the peaks of the investigated compounds was carried out by comparison of their retention times with those obtained injecting standards in the same conditions, as well as by spiking *Agaricus blazei* samples with stock standard solutions. At least nine compounds could be identified and quantified. The nucleotides were UDP (0.60±0.01 nmol/mg extract) and AMP (0.94±0.02 nmol/mg). The nucleosides were guanosine (2.96±0.02 nmol/mg), adenosine (14.40±0.38 nmol/mg), cytidine (2.00±0.13 nmol/mg) and uridine (10.82±0.24 nmol/mg). And, finally, the nucleobases were uracil (1.39±0.02 nmol/mg), hypoxanthine (0.72±0.04 nmol/mg) and xanthine (2.36±0.09 nmol/mg). For testing the purinergic activity of the *Agaricus blazei* extracts, the isolated perfused rat liver was used, a system that has been shown to respond in a characteristic way to purinergic agents. The once-through perfusion system was used and the perfusion fluid was the Krebs/Henseleit-bicarbonate buffer (pH 7.4), saturated with a mixture of oxygen and carbon dioxide (95:5%) and equilibrated to 37°C. Extracts were infused at concentrations up to 400 mg/liter, corresponding to total concentrations of nucleotides and nucleosides of up to 15 μM. The extract produced transient increases in the portal and arterial perfusion pressure. In livers from fed rats, with a high glycogen content, the extract increased the glycometabolic glucose release in a transitory manner. It also caused a transient increase in lactate production and a relatively stable increment in oxygen uptake. In livers from fasted rats perfused with lactate (2 mM) as the gluconeogenic precursor the extract caused a transient decrease in oxygen uptake followed by stimulation and a transient decrease in glucose synthesis. These and other effects have been reported to occur in the perfused rat liver upon adenosine and AMP infusion, for example. It has also been reported that these effects are, partly at least, Ca²⁺-sensitive and dependent on cociosanoid release from the Kupffer and endothelial cells. In order to test this possibility for the *Agaricus blazei* extract, experiments were done in which Ca²⁺ was omitted from the perfusion fluid. Under these conditions most effects were nearly abolished or at least substantially reduced. The effects were also sensitive to two different inhibitors of cociosanoid synthesis, namely indomethacin and bromophenacyl bromide. The effects were also sensitive to suramin, a known antagonist to purinergic receptors. The results obtained in the present work allow to conclude (1) that nucleotides, nucleosides and nucleobases are important components of the fruiting bodies of *Agaricus blazei* and (2) that extracts containing these compounds are in fact able to exert many effects that are characteristic of the purinergic action in the liver. The effects of the extract will probably be more complex that those of each isolated substance due to the possibility of synergism, a phenomenon that can always be expected from complex mixtures.

**Keywords:** *Agaricus blazei*; extracts; nucleosides; nucleotides; purinergic action.

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QuantiFERON-TB GOLD ELISA assay for the detection of *Mycobacterium tuberculosis* specific antigens in blood specimens of HIV positive patients in a high burden country

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Worldwide, tuberculosis is a life-threatening infection. Despite improvements in therapy it results in 2 M deaths and 9 M new cases annually. This study evaluated the use of the QuantiFERON-TB GOLD ELISA assay in a high HIV/TB burden setting in an ARV clinic at the Tshwane District Hospital, South Africa. The sensitivity and specificity of the QF assay in the clinic were 30% (9/30) and 63% (19/30) respectively when compared to the gold standard culture results. Analysis also suggested that the sensitivity of the QuantiFERON assay is determined by a limiting patient CD4 value beyond the simple role of metabolic intermediates. In mammals this group of compounds is involved in the regulation and modulation of various physiological processes mediated by purinergic receptors, so that they could be useful to the attempts of explaining the physiologic and pharmacological effects of this species. Taking this into account the present work had two main purposes: (1) to identify and quantify the nucleotides, nucleosides and nucleobases of *Agaricus blazei* hydroalcoholic extracts; (2) to test the extract for a possible purinergic action due to the presence of those compounds. Identification and quantification were done by means of high-performance liquid chromatography (HPLC). Samples of the fruiting bodies (basidiocarps) of *Agaricus blazei* were extracted with ethanol 70% and the extracts were freeze-dried and dissolved in water for analysis. The HPLC system (Shimadzu, Japan) consisted of a SCL-10AVP system controller, two model LC10ADVP pumps, a model CTO-10AVP column oven and a model SPD-10AVP UV-VIS detector. A reversed-phase C18 HRC-ODS column (5 μm; 150x6 mm ID; Shimadzu, Japan) protected with a GHRC-ODS pre-column (5 μm; 10x4 mm ID, Shimadzu, Japan) was used with a gradient from reversed-phase 0.044 mol/L phosphate buffer solution, pH 6.0, to 0.044 mol/L phosphate buffer solution plus methanol (1:1), pH 7.0, at 0.8 mL/min. The identification of the peaks of the investigated compounds was carried out by comparison of their retention times with those obtained injecting standards in the same conditions, as well as by spiking *Agaricus blazei* samples with stock standard solutions. At least nine compounds could be identified and quantified. The nucleotides were UDP (0.60±0.01 nmol/mg extract) and AMP (0.94±0.02 nmol/mg). The nucleosides were guanosine (2.96±0.02 nmol/mg), adenosine (14.40±0.38 nmol/mg), cytidine (2.00±0.13 nmol/mg) and uridine (10.82±0.24 nmol/mg). And, finally, the nucleobases were uracil (1.39±0.02 nmol/mg), hypoxanthine (0.72±0.04 nmol/mg) and xanthine (2.36±0.09 nmol/mg). For testing the purinergic activity of the *Agaricus blazei* extracts, the isolated perfused rat liver was used, a system that has been shown to respond in a characteristic way to purinergic agents. The once-through perfusion system was used and the perfusion fluid was the Krebs/Henseleit-bicarbonate buffer (pH 7.4), saturated with a mixture of oxygen and carbon dioxide (95:5%) and equilibrated to 37°C. Extracts were infused at concentrations up to 400 mg/liter, corresponding to total concentrations of nucleotides and nucleosides of up to 15 μM. The extract produced transient increases in the portal and arterial perfusion pressure. In livers from fed rats, with a high glycogen content, the extract increased the glycometabolic glucose release in a transitory manner. It also caused a transient increase in lactate production and a relatively stable increment in oxygen uptake. In livers from fasted rats perfused with lactate (2 mM) as the gluconeogenic precursor the extract caused a transient decrease in oxygen uptake followed by stimulation and a transient decrease in glucose synthesis. These and other effects have been reported to occur in the perfused rat liver upon adenosine and AMP infusion, for example. It has also been reported that these effects are, partly at least, Ca²⁺-sensitive and dependent on cociosanoid release from the Kupffer and endothelial cells. In order to test this possibility for the *Agaricus blazei* extract, experiments were done in which Ca²⁺ was omitted from the perfusion fluid. Under these conditions most effects were nearly abolished or at least substantially reduced. The effects were also sensitive to two different inhibitors of cociosanoid synthesis, namely indomethacin and bromophenacyl bromide. The effects were also sensitive to suramin, a known antagonist to purinergic receptors. The results obtained in the present work allow to conclude (1) that nucleotides, nucleosides and nucleobases are important components of the fruiting bodies of *Agaricus blazei* and (2) that extracts containing these compounds are in fact able to exert many effects that are characteristic of the purinergic action in the liver. The effects of the extract will probably be more complex that those of each isolated substance due to the possibility of synergism, a phenomenon that can always be expected from complex mixtures.

**Keywords:** Tuberculosis, QuantiFERON-TB GOLD, HIV positive patients
Quantitative evaluation of lactobacilli and \textit{Clostridium difficile} in faeces of patients with antibiotic associated diarrhoea and antibiotic resistance of isolated strains

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Background. \textit{C. difficile} is the most frequently recognized agent of antibiotic associated diarrhoea in hospitalised patients. The \textit{C. difficile} infections can vary from asymptomatic colonization to severe colitis. Some previous studies have shown protective role of intestinal lactobacilli against \textit{C. difficile} colonization/infection. During recent years some new emerging hypervirulent ribotypes which are highly resistant to newer fluoroquinolones have been recognized in several countries.

The aims of our study were (1) to evaluate quantitative counts of \textit{C. difficile} in infected patients; (2) to correlate counts of intestinal lactobacilli and the presence of free \textit{C. difficile} toxin in faeces with the counts of \textit{C. difficile} and (3) to detect antibiotic sensitivity and ribotype of isolated \textit{C. difficile} strains.

Methods. Faecal samples (n=34) were collected from antibiotic associated diarrhoea patients from Estonian hospitals during 2008. Samples were analyzed for \textit{C. difficile} toxins, quantitative counts of \textit{C. difficile} and lactobacilli (colony forming units per gram, log CFU/g). Isolated strains of \textit{C. difficile} were ribotyped by PCR and antimicrobial susceptibility was tested by E-tests (minimal inhibitory concentration, MIC).

Results. From 34 investigated samples 21 were toxin and/or \textit{C. difficile} culture positive. Altogether 20 \textit{C. difficile} strains were isolated. Counts of \textit{C. difficile} varied between 5.3 and 7.9 log CFU/g in infected patients (median 7.0). There was trend of higher \textit{C. difficile} counts in patients with positive direct toxin test compared with negative ones (medians 7.3 vs 6 log CFU/g), however this was not statistically significant (p=0.076). Lactobacilli were detectable in 8 samples out of 34 with counts 0-4.58 log CFU/g (median 0). There was no difference between presence or counts of lactobacilli in \textit{C. difficile} infected or non-infected patients. The MIC values (mg/L) of \textit{C. difficile} strains were following: metronidazole 0.032-1 (median 0.064), vancomycin 0.25-4 (0.5), erythromycin 0.125-256 (0.38), clindamycin 0.25-256 (1.5), moxifloxacin 0.25-1 (0.5), levofloxacin 1-32 (2). Six strains resistant to fluoroquinolones belonged to ribotypes 017; 012; 095; 046; 012 and 001. No hypervirulent ribotypes (027, 078) were detected.

Conclusions. Despite of high resistance to fluoroquinolones of some \textit{C. difficile} strains, no hypervirulent ribotypes were found. Metronidazole and vancomycin MICs were usually low, thus these antibiotics could be used for treatment of \textit{C. difficile} infection. There was trend of higher \textit{C. difficile} counts in toxin positive samples.

Keywords \textit{C. difficile}, hypervirulent ribotypes, antibiotic resistance, intestinal lactobacilli

Recombinant protein of \textit{Entamoeba histolytica} expressed by \textit{E. coli} is recognized by antibodies from patients with invasive amebiasis

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Amebiasis is the second cause of deaths due to parasites worldwide. The commercial immunotests for diagnosis this disease don’t have enough accuracy because antibodies directed against the amoebas that circulate among endemic zones populations, another one is the high enzymatic \textit{E. histolytica} content, which causes degradation of the amoebic extracts. Enzyme inhibitors are used worldwide to diminish the activity of the amoebic proteases. When proteins degrade, they also lose their antigenicity; therefore these extracts are not good enough to be used as a diagnostic test base. Our group succeeded in preserving the antigenic molecules without using enzymatic inhibitors. These molecules can be recognized by the antibodies of patients with invasive amebiasis. (USA patent No. 5459042 and Mexican patent No. 209646). Immunological tests were designed using these antigens. One of this is a Western Blot test (USA patent No.5861263 and Mexican patent No. 209648). The clinical utility of WB test was valued testing sera from patients with diverse liver diseases and 124 patients with invasive amebiasis. WB was compared with the commercial hemagglutination indirect test (IHA) The following clinical utility parameters were obtained: Sensibility (WB 99% IHA 82%), Specificity (WB 100% IHA 100%), predictive positive value (WB 100% IHA 100%), predictive negative value (WB 98% IHA 88%) and accuracy (WB 99% IHA 88%). Under the same conditions, the WB showed a better diagnostic performance. The WB pattern is constituted by six antigenic bands, among them there is one immunodominant band of low molecular weight named by us as “BPM”. In this study we produced a recombinant BPM protein. The terminal amino sequence of “BPM” was determined and obtained the cDNA that codifies for \textit{E. histolytica} “BPM” protein. Recombinant BPM was expressed by induction of PET28a-transformed \textit{Escherichia coli} BL21 cells. Gene codifying “BPM” was fused to a stretch of six histidine residues. Purified recombinant “BPM” antigenicity was analyzed by SDS-PAGE and WB using sera of patients with invasive amebiasis. Sera from uninfected healthy donors were used as controls. Also we implemented a Dot Blot to compare recombinant BPM with the BPM produced by \textit{E. histolytica}. The recombinant BPM is recognized only by antibodies from patients with invasive amebiasis. We will use the recombinant BPM at implementation of a diagnostic quick test.

Keywords \textit{E. histolytica}, hypervirulent ribotypes, antibiotic resistance, intestinal lactobacilli

Grants: PAICYT CN1575-07 SEP CONACYT 50310 Clave 25617
Rheological Characterisation and Effect of Abiotic Factors on the Antimicrobial Efficacy of Chitosan - Based Hydrogels Containing Alpha-Hydroxy Acids

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The objective of this study was to investigate the rheological properties and antibacterial efficacy of chitosan/alpha-hydroxy acids (lactic acid and glycolic acid) and cellulose polymers, both in hydrogels, in order to produce a formulation with improved activity against Propionibacterium acnes and Staphylococcus aureus, which can potentially be used in the treatment of acne.

The rheological characterisation of the hydrogels was examined using continuous shear and viscoelastic creep. The antibacterial activities of formulations were performed by the well diffusion and broth microdilution. The hydrogels formulated with only chitosan showed pseudoplastic behaviour while the chitosan hydrogels with cellulose polymers presented viscoelastic properties. The antibacterial activity was proportional to AHA and chitosan concentration. It was enhanced at low pH values and with high molecular weight chitosan and did not change with the incorporation of two cellulose polymers. The antibacterial mechanism of chitosan has currently been hypothesized as being related to surface interference.

The results show that chitosan-based hydrogels containing AHA and cellulose polymers are viscoelastic, indicating good applicability onto the skin, and they present bacterial activity under various experimental conditions.

Screening and evaluation of human intestinal lactobacilli for the development of novel gastrointestinal probiotics


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Lactobacilli have been used as probiotics in food products and dietary supplements for decades. Today, there is increasing interest in developing novel genetically engineered probiotics, e.g. the application of lactobacilli as vehicles for delivery of both active and passive immunity.

The aim of this study was to screen intestinal lactobacilli strains for their beneficial properties to select those that could be used for development of novel genetically engineered probiotics.

Methods. Ninety-three human intestinal Lactobacillus isolates were subjected to screening procedures. They were identified using an API 50 CHL kit and ITS-PCR analysis, examined for auto-aggregation, tolerance of low pH, bile and pancreatin content, antibiotic susceptibility, and haemolytic activity. Six potential probiotic strains were selected and examined for safety in a mouse model.

Results. The investigated lactobacilli strains belonged to 11 species of all three fermentation types (44 obligately homofermentative, 28 facultatively heterofermentative and 21 obligately heterofermentative strains). Fifty-nine percent of the examined lactobacilli showed the ability to auto-aggregate, 97% tolerated a high concentration of bile (2% w/v), 50% survived for 4 h at pH 3.0, and all strains were unaffected by a high concentration of pancreatin (0.5% w/v). One Lactobacillus buchneri strain resistant to tetracycline was excluded from further studies. None of the tested strains caused the lysis of human erythrocytes. Five of 6 strains caused no translocation in animal model, and were considered safe.

Conclusions. The present study identified several strains that have properties required for a potential probiotic strain. In the next phase of the characterization, human trials are necessary to study their survivability in human gut and confirm their safety in humans. Our results can be applied for further studies to design a genetically engineered probiotic product.

Keywords: Lactobacillus; Probiotics; Acid tolerance; Bile tolerance; Antibiotic susceptibility; Haemolytic activity; Safety
Screening of antimicrobial activity of several extracts of *Cistus ladanifer* and *Arbutus unedo*

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Plants contain numerous biologically active compounds, many of which have antimicrobial properties and can be seen as sources of agents to combat microbial diseases. The increase in the number of infections in the population and of infectious agents resistance to antibiotics, has enhanced interest in plant extracts or plant-derived compounds, which may represent an alternative to current antimicrobial agents and provide valuable sources of new medicinal agents. For this reason, natural products are being screened for potential use in the therapy of bacterial and fungal infections. To contribute to these studies, we investigated the *in vitro* antimicrobial activity of different crude extracts obtained from *Cistus ladanifer* and *Arbutus unedo*. *C. ladanifer*, known as rock-rose is a natural aromatic plant of the Iberian Peninsula. All the *Cistus* species are frequently used in traditional medicine for their antimicrobial, antitumor, antiviral and anti-inflammatory properties. *A. unedo*, known as strawberry tree, belongs to the Ericaceae family and is native to the Mediterranean climate. The leaves of *Arbutus unedo* L. are diuretic, urinary antiseptic, antidiarrheal, astringent, deparative, against blennorrhagia and as an antihypertensive. The ethanol, methanol and acetone/water extracts of *Cistus ladanifer* and *Arbutus unedo* L. were extracted using different extraction methods and their antimicrobial properties were investigated against reference strains including three Gram-positive (*Bacillus cereus*, *Enterococcus faecalis* and *Staphylococcus aureus*), four Gram-negative (*Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Salmonella typhimurium*) and three yeasts (two strains of *Candida albicans* and one of *Candida tropicalis*) and against clinical isolates of *Helicobacter pylori* and methicillin-resistant *Staphylococcus aureus*. The various extracts were tested using the standard M2 and M44 disc diffusion method of the National Committee for Clinical Laboratory Standards (NCCLS), followed by the agar dilution method that was used to determine the minimum inhibitory concentration to the strains presenting susceptibility to various extracts of *C. ladanifer* and *A. unedo*. All the extracts inhibited more than one microorganism, presenting antimicrobial activity against Gram-positive bacteria, *Klebsiella pneumoniae*, *Candida tropicalis* and *Helicobacter pylori*. All the different extracts from the tested plants showed inhibitory effects against most tested microorganisms, including a significant in vitro effect against *H. pylori*. These inhibitory effects could be considered relevant to the development of new agents for inclusion in the treatment or prevention of infections by the tested strains as *H. pylori*.

**Keywords** Antimicrobial activity, *Cistus ladanifer*, *Arbutus unedo*

Sensitivity of microorganisms on silver nanoparticles.

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Recently we have seen great progress in nanotechnology and their integration with biology. Among the most promising nanomaterials with antibacterial properties are metallic nanoparticles, which show increased chemical and biological activity due to their surface volume ratios. Silver has been known for a long time as strong antimicrobial agent. We synthesized different silver silica spheres by sol-gel (the final product is a powder) and marked their antimicrobial activity (directly as powder and indirect as of textiles surface) [1, 2]. The following bacteria strains were tested: *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 11229 and *Klebsiella pneumoniae* ATCC 4352. We tested also fungus strains: *Candida albicans* ATCC 10231. As opportunistic pathogens, *Klebsiella* spp. primarily attack immunocompromised individuals. The high rate of nosocomial *Klebsiella* colonization appears to be associated with the use of antibiotics rather than with factors connected with delivery of care in the hospital. The virulent strains of *E. coli* cause gastroenteritis, urinary tract infections and neonatal meningitides. Clinical diseases associated with *S. aureus* are pyodermas, folliculitis, osteomyelitis, pneumonia, arthritis, bacteremia, septicemia and the scalded skin syndrome. The application of the silver-based compounds used in catheters, tracheostomies, dressings and medical clothes may be helpful in prevention of the infections caused by those bacteria. As precursor of silver were used ions of silver nitrate (AgNO₃) or silver nitrate (AgNO₃). Process synthesis of silver/silica nanocomposites was two-step procedure: first we impregnated silica spheres into silver solution and next reduced silver ions to metals nanoparticles (as shown in graph). Nanoparticles were characterized by electron microscope (TEM/SEM), X-ray diffraction (XRD), spectra UV-VIS. Antibacterial and antifungal effect was determined by MIC and MBC/MFC values according to reference methods CLSI (NCCLS) [3, 4] and/or determined inhibited zone according to standard ISO/DIS 20645:2002 [5]. Microorganisms shown high sensitivity on silver nanoparticles, both powder and surface textiles. *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 11229 and *Klebsiella pneumoniae* ATCC 4352 indicate similar sensitivity to silver included in polymer paste.

This work was supported by the Ministry of Science and Higher Education (Grant 4210/PB/BiG/09). Graph 1. Silver (20 nm) on the silica surface (TEM)

**Keywords** sol-gel, nanotechnology, nanoparticles, silver, silica, microorganisms

**References**

Serotype Distribution of Chlamydia trachomatis Isolated from clinical urogenital samples in North-Eastern Croatia

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The fast molecular diagnostics of Chlamydia trachomatis infections and adequate therapy of the infected individuals are the crucial step in the C. trachomatis spread control. Due to the chronic and "silent" infection and variable antigenic structure of the C. trachomatis we supplemented Direct Immune Fluorescence with the molecular method RT-PCR. The aim of the present study has been to reveal the most prevalent serotype of the C. trachomatis detected in urine and gynaecological samples and to monitor the infection and the therapy efficiency. The determined serotype distribution has been compared with the C. trachomatis distribution pattern in other regions of the World. COBAS TaqMan CT test is an in vitro nucleic acid amplification test which utilizes real time PCR technology. The test has been developed to confirm analogous detection of all 15 C. trachomatis serotypes. Subsequently all the positive samples have been analysed directly by sequencing of the amplified omp1 fragments using Applied Biosystems 3130 Genetic Analyser. Genotyping and sequence mutation analysis have been performed using ABI SEQUENCE software and compared with the determined sequences of all known C. trachomatis serotypes.

The preliminary study determined serotype E (in agreement with Sweden and Taiwan data) as the dominant one, followed by F, K, G, D, B, J and Ia (differs Sweden and Taiwan data). Further investigation and data analysis are in progress.

Keywords: urogenital infections, C. trachomatis diagnostics, serotypes, sequencing

Simple and efficient method of E. coli disruption for recombinant pneumococcal surface protein A production

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Streptococcus pneumoniae is a pathogenic bacterium responsible for million deaths of children, elderly and immune compromised people worldwide. New conjugated vaccines are being developed using as carriers pneumococcal surface proteins, which are more conserved than pneumococcal polysaccharides and would offer a broader coverage. The pneumococcal surface protein A (PspA) was chosen for the development of a new vaccine as it has been demonstrated to be one of the most important virulence factors of S. pneumoniae. A recombinant fragment of PspA from clade 3 (rfpPspA3) was produced in high cell density culture of E. coli as intracellular soluble protein and the first step for its recovery is the cell disruption. Mechanical cell disruption is a general method of choice and at small-scale French press is normally used, while at bench and large-scale equipment as continuous high-pressure homogenizer or bead mill is required. A controversy exists in the literature about the mechanism of cell disruption by high-pressure homogenization and several studies have demonstrated correlations between physical parameters and cell disruption, but no simple protocol for cell disruption is available. In the present work, the cell disruption was addressed for the development of an industrial production process of rfpPspA3.

E. coli BL21(DE3) harboring pC737b+rfpspA3 was grown in 5-L bioreactor using high cell density medium (HCD) with glucose or glycerol as carbon source. Fed-batch cultures were carried out with exponential feeding of the carbon source in order achieve high cell density. The induction was performed with 0.5 mM IPTG + 20g/L lactose. The cells were centrifuged at 17.969 g for 30 min at 4°C and stored at -20°C. For disruption, 100g cell wet weight was resuspended with 1.0 L 25mM Tris pH 8.0 + 0.1% Triton X-100 + 1.0mM phenylmethylsulfonyl fluoride (protease inhibitor). The cells were disrupted in a continuous high pressure homogenizer (APV Gaulin 60) at 60MPa and flow rate of 1L/min. The cell suspension reservoir was jacketed, a tube and shell heat-exchanger was placed immediately after the homogenizer outlet and a circulating cooling solution at 4°C was employed in order to keep the temperature under 20°C. The cell suspension was continuously recirculated in closed loop through the homogenizer for 10 min and samples were taken for viable count on LB/agar (CFU/mL) and determination of optical density at 600nm (OD). For protein and rfpPspA3 quantification, 2.0 mL of sample was centrifuged at 20,817 g for 1.0 h at 4°C and the supernatant was used. The total protein content was determined by Lowry and rfpPspA3 was quantified by densitometry of SDS-PAGE bands (densitometer GS-800 and software Quantity One 4.6.3, BioRad). The efficiency was calculated as a percentage of the OD obtained after solubilization of cells with 0.1M NaOH.

After 6 min for cells produced with glucose as carbon source and 7 min for cells produced with glycerol as carbon source, the OD stabilized and the disruption efficiency was calculated as 96.7% and 92.7%, respectively. However, the viable count of homogenate from glycerol culture reached a plateau after 9 min. The presence of the detergent Triton X-100 in the lysis buffer together with the freeze/thaw process was responsible for the rfpPspA3 release to the supernatant before the homogenization: 1.8 mg/mL rfpPspA3 from cells produced with glucose and 1.0 mg/mL from cells produced with glycerol. At the end of homogenization, it was obtained 97.5% reduction in OD (from 22 to 0.55) for the homogenate from glycerol culture and 94.4% in OD (from 10 to 1.52) for the homogenate from glucose culture, and 4-log reduction in viable count for cells from both cultures: from 9.8x106 to 10 CFU/mL for glycerol and from 4x105 to 9x104 CFU/mL for glucose. The results indicated glycerol could be acting as cryoprotector and avoiding cell lysis during freeze/thaw process. There was no differences in total protein content (~11 mg/mL) and in target protein (~3.75 mg/mL) released from cells produced using glycerol or glucose after 6-7 min homogenization.

In conclusion, a simple and straightforward method for E. coli disruption was established for intracellular rfpPspA3 recovery; it consisted of 10 minutes of continuous flow through a high-pressure homogenizer in a closed loop system, without any interruption for lowering the temperature, since it was kept under 20°C using a refrigerated reservoir in the inlet and a heat-exchanger in the outlet of the equipment. High-pressure homogenizer is much simpler to use than French press and has the advantage of being easy to scale-up, because nowadays there are small and large scale equipments that allow to process volumes from hundred milliliters up to hundred liters.

Financial support: FAPESP.

Keywords: recombinant pneumococcal surface protein A; cell disruption of E. coli
Stempholactoside, new Stemphol derivative isolated from tropical endophytic fungus *Gaeumannomyces amomi* BCC4066

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We have investigated a group of novel strains which were identified as potential producers of polyketides, using PCR based gene mining. The aim of this work was to study the diversity of reducing type I polyketide synthase and isolate polyketide molecules. Cultures grown to investigate the production of these important natural products were also investigated for other metabolites. We first studied the diversity of reducing type I polyketide synthase in these species using a molecular and bioinformatics approach. All strains were cultivated in preferable medium and column chromatography used for separation. Structure elucidation was analyzed by spectroscopic method. The ethyl acetate extract of the cultures of *Gaeumannomyces amomi* BCC4066, an endophytic fungus found in healthy parts of ginger or Thai name: Kha-pa (*Alpinia malaccensis*) have been isolated by fractionation. A new stemphol derivative named stempholactoside (1), together with three known metabolites, indole-3-carboxylic acid (2), stemphol (3) and kojic acid (4) have been elucidated. The structure of 1 was established by spectroscopic methods, including 2D-NMR experiments (COSY, HMQC, and HMBC), and GC/MS. Stemphol displayed significant growth inhibitory activity against the brine shrimp (*Artemia salina*) at a concentration of 10 µg/ml.

Keywords: polyketide synthase, new stemphol, natural product, ascomycete fungi

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Streptococcus intermedius trigger quorum-sensing genes in *Porphyromonas gingivalis*

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Quorum sensing is a genetic regulation in response to cell density that can regulates various functions in bacteria. Among them we can find various cell functions associated with secondary metabolism, biofilm formation and virulence gene expression. This phenomenon was discovered in the marine bacteria *Vibrio fischeri* and *Vibrio harveyi* who produce autoinducers, and when their concentration reaches a threshold level in environment, they bind to a response regulator protein and induce expression of luciferase gene cluster. Today, we can recognized two kind of quorum-sensing systems in *V. harveyi* one utilizes an acylhomoserine lactone as the signal and other uses a *luxS* gene homologue that might be implicated in not only in a quorum-sensing system but also in stress gene regulation. In this study, we report the regulation of gene expression in *P. gingivalis* as response to different cell concentration of *Streptococcus intermedius*, another natural oral habitant. Both bacteria were incubated at next ratios: 1:1, 1:10 and 10:1 of *P. gingivalis*: *S. intermedius*. The genetic expression of the locus PG0538, PG0533 and PG1230 was evaluated with qPCR. Values were compared with those obtained with cultures of *P. gingivalis* pure and the 1:1 *P. gingivalis*: *S. intermedius* relationship. Genetic regulation was clear when *P. gingivalis*: *S. intermedius* had a ratio of 1:10, here the locus PG0538 was down regulated while PG0533 was up regulated. However, when the ratio was of 10:1 *P. gingivalis*: *S. intermedius*, the real value remains unchanged. It is well known that in the oral cavity live many microorganism and their cell concentrations and cell ratio are in constant fluctuation. Early reports have been suggested that *S. intermedius* are associated with periodontitis too. In this sense, we addressed our strategy to search how *P. gingivalis* could to response to the presence of another oral microorganism. Our results showed that the quorum sensing system could to regulate genetic systems as response to another microorganism.

Key words: Porphyromonas gingivalis, Streptococcus intermedius, quorum sensing, qPCR
Structural Conservation of Helicobacter hepaticus Catalase and Potential Contributions to Autoimmunity

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Background: Helicobacter hepaticus colonizes the mouse intestine and has been associated with hepatic inflammation and neoplasia in susceptible mouse strains. In this study, we characterized the catalase of an enteric bacterial H. hepaticus for the first time. H. hepaticus catalase is a highly conserved enzyme that may be important for bacterial survival in the mammalian intestine. Methods: Recombinant H. hepaticus catalase was expressed in E. coli to study the enzymology in vitro. The H. hepaticus catalase sequence was compared with diverse bacterial and mammalian catalases. To study auto-reactive immune responses to H. hepaticus and endogenous mouse catalases during the course of H. hepaticus infection, two groups of mice (H. hepaticus-infected and uninfected C57Bl/6 IL-10-deficient mice) were evaluated by immunoblotting with recombinant antigens. To correlate the mouse model data to human disease, sera from patients with primary sclerosing cholangitis (PSC) were assessed. Results: H. hepaticus catalase contains a highly conserved heme-ligand domain and conserved surface-predicted motifs that are shared with mammalian catalases. Mice experimentally infected with H. hepaticus (n=8) demonstrated humoral immune responses to murine and H. hepaticus catalases. None of the uninfected mice demonstrated antibody responses to either catalase. Human patients with primary sclerosing cholangitis (PSC) demonstrated antibodies to human and H. hepaticus catalases, indicating that human immune responses may also recognize endogenous human catalase as a consequence of chronic inflammation. Control human sera lacked reactivity to catalase. Conclusions: Catalases are highly conserved enzymes in bacteria and mammals, highlighting their potential roles in microbial:host interactions. The cross-reactive immune responses to mammalian catalases suggest the potential role of H. hepaticus catalase to contribute to auto-reactive immune responses in the hepatobiliary and gastrointestinal tracts. Finally, vaccination strategies may benefit from the application of conserved and divergent microbial antigens to broad-spectrum immunoprotection.

Study of the influence of ethylene oxide sterilization variables on Bacillus subtilis inactivation

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Introduction
Nowadays, ethylene oxide (EO) is a dominant sterilization agent used in medical device industry due to its effectiveness and compatibility with most materials, together with the technical and technological advances that allow overlapping difficulties associated to this agent.

Aim
This work intends to study the influence of the process variables, e.g., temperature (T), ethylene oxide (EO) concentration and relative humidity (RH), on Bacillus subtilis inactivation, aiming to provide a predictive model of integrating lethality.

Material and Methods
Experiments were carried out in an EO sterilizer with controlled temperature, EO concentration and humidity. The sporidical activity of a specific EO sterilization cycle was assessed by recover and enumeration of bacterial viable spores from B. subtilis biological indicators.

Results
Results showed that temperature and EO concentration were the most significant factors affecting the inactivation kinetics of Bacillus subtilis. Mathematical relations describing the influence of the referred process variables on microbial inactivation kinetics were successfully developed and achieved the final inactivation kinetic model:

\[
\log\left(\frac{N}{N_0}\right) = -7.5 \exp\left(\frac{-\left[1.42 \times 10^{-4} T - 4.96 \times 10^{-3}\right] + 5.54 \times 10^{-8} T - 1.25 \times 10^{-6} [\text{EO}]^2}{-7.5} \right) \\
\times \left[1.63 \times 10^4 - 1.06 \times 10^3 \ln[\text{EO}] + \left(-1.25 \times 10^2 + 8.23 \times 10^3\right) - 1\right] + 1
\]

where N is the microbial load at a particular process time t (the index 0 is related to initial microbial load).

Conclusion
An inactivation model that described the process kinetics only in terms of the relevant process variables (temperature and EO concentration) was achieved. The predictive ability of this integrated model was assessed, and its adequacy in predicting B. subtilis inactivation was verified.

The results of this work are certainly a contribution for an efficient control model, design and optimization of the EO sterilization process.

Keywords Modelling, Bacillus subtilis, ethylene oxide, sterilization
Synergistic Antimicrobial Activity Among Hydroalcoholic Extract of Leaves of Trees in the Brazilian Territory Common

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The use of medicinal plants in the world, and especially in South America, contributes significantly to primary health care. Many plants are used in Brazil in the form of crude extracts, infusions or plasters to treat common infections without any scientific evidence of efficacy. This study aimed to evaluate the antimicrobial activity of hydroalcoholic extract from leaves of Eugenia uniflora, Punica granatum and association between the extracts against Staphylococcus aureus (ATCC25923). The antibacterial activities of extracts were determined by macrodilution techniques in BHI broth. The minimum inhibitory concentration (MIC90) was determined by measuring the optical density in the spectrophotometer (540 nm) and was defined as the lowest concentration of crude extract that produced a 90% reduction in visible growth compared with control (non inoculated broth). All concentrations of extracts and association of extracts from the leaves of the plants tested showed some inhibition of bacterial growth. However, only the combination of the extracts was obtained MIC90. The greatest inhibition obtained by the extract of the leaves of plants Eugenia uniflora and Punica granatum on the growth of Staphylococcus aureus was 88% and 74%, respectively. The MIC90 for the combination of extracts of two plant species was 0.5 mg/mL. Inhibitory activity on bacterial growth of the association of the hydroalcoholic extract from leaves of Punica granatum with Eugenia uniflora was higher when compared with the inhibition of bacterial growth promoted by extracts alone, indicating a bacteriostatic synergistic effect between these two extracts. Acknowledgments: FAPESP

Keywords: Medical plants; antimicrobial activity; sinergism

Syzygium aromaticum (clove) extract reduce virulence factors mediated by QS in Gram negative bacteria

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The emergence of antibiotic resistance strengthens the need for novel therapeutic drugs. It has been suggested that the inhibition of Quorum-Sensing (QS) offers an alternative to antibiotic mediated bactericidal or bacteriostatic approach and reduces the risk for development of resistance. QS is an important process regulated by molecules named autoinducers (AI) involved in bacterial survival, biofilm formation, motility, swarming and secondary metabolite production. In Gram-negative organisms. N-acyl-homoserine lactones (AHL) are the most common group of AI molecules.

The antimicrobial effects of herbs and spices have been documented and used in herbal medicine in many countries but their ability to inhibit QS as poorly studied. Eighteen methanolic extracts from herbs and spices was tested to determinate their ability to inhibit QS in Chromobacterium violaceum, Salmonella Typhimurium and Pseudomonas aeruginosa PAO-1.

Only Syzygium aromaticum (clove) extract reduced AHL and violacein production from C. violaceum, Salmonella invasivity, and alginate synthesis and swarming motility in PAO-1. Eugenol is the mayor constituent of clove extract (75-90%). However, eugenol do not exhibit anti-QS activity. The identification of anti-QS phytoconstituents from “clove” is needed to assess the mechanism of action against these Gram-negative bacteria.

Keywords: Quorum-sensing, Gram-negative, clove
The use of real-time PCR assays for the detection, identification and drug susceptibility patterns of *Mycobacterium* in sputum and blood specimens in HIV positive patients

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Rapid detection of *Mycobacterium* spp are essential, since patients are often infected with *Mycobacterium* spp other than *M. tuberculosis*, such as *M. avium* and *M. kansasii*. Bacteriological culture is considered the diagnostic gold standard to identify *Mycobacterium tuberculosis* but results are only available between 4 to 8 weeks. The diagnosis of MDR and XDR-TB takes an additional 2 to 3 weeks. Molecular assays, such as real-time PCR and GenoType® MTBDR plus have proven to be rapid and accurate to identify *Mycobacterium* spp. *Mycobacterium tuberculosis* drug resistant strains are mainly due to mutations in genes encoding drug target or drug converting enzymes. The katG and inoh are the most frequently associated with isoniazid resistance. Mutations, in Rif resistant strains, confined to a short 81 bp DNA region encodes for the β-subunit of the RNA polymerase. More than 83% of the Rif-resistant isolates are also resistant to INH. The introduction of rapid molecular testing for all acid-fast bacilli positive cases for routine diagnostics has the potential to aid in the rapid commencement of treatment to prevent the spread of MDR-TB.

A total of 60 human immunodeficiency virus (HIV) positive patients, older than 18 years of age, attending the antiretroviral (ARV) clinic at Tshwane District Hospital, clinically presenting with TB symptoms and who has given informed consent were randomly selected over a six month period (October 2008 to March 2009) and recruited for this study. Both blood and sputum specimens were collected and transported to the Diagnostic Division at the Department of Medical Microbiology, University of Pretoria. Routine analysis such as sputum microscopy and culture were performed. The remainder of the specimens was transported to the Research Division at the Department of Medical Microbiology, University of Pretoria where real-time PCR assays were performed to detect *Mycobacterium* spp as well as determine the prevalence of INH and Rif resistant genes.

The real-time PCR assay identified 28% (17/60) *M. tuberculosis* (Tm = 55-57°C preliminary), 2% (1/60) *M. kansasii* (Tm = 59-62°C preliminary) and 70% (42/60) of the isolates *Mycobacterium* spp negative. No *M. avium* were detected. The 17 *M. tuberculosis* positive specimens were further used to detect INH and Rif resistance genes. All 17 specimens had either no mutation or one or more mutations at the specific gene targets (rpoB, rpsL, katG and inhA).

The conventional methods used to detect *M. tuberculosis* and determine the resistance patterns are prolonged and laborious methods when compared to real-time PCR. The PCR amplification process can be completed in 2 to 4 hours after obtaining the processed clinical specimen with an additional 2 to 24 hours for the detection of mycobacteria resulting in quicker diagnosis compared to the 4 to 8 weeks when cultured. Molecular assays are potentially the most sensitive method for the identification and detection of drug resistance strains and are theoretically able to provide a same-day diagnosis from clinical samples. This will ensure that patients can go home with the correct treatment and will not be lost to the system.

Variation in IL-8 release and ICAM-1 expression by different human lung airway epithelia induced by *Burkholderia cepacia* complex (Bcc)

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*Burkholderia cepacia* complex (Bcc) is a group of Gram negative pulmonary pathogens associated with life-threatening infections in patients with cystic fibrosis (CF). The airway epithelium plays a crucial role in the initiation and modulation of inflammatory responses to these pathogens. Interleukin (IL)-8 is a potent chemoattractant for neutrophils and the expression of intercellular adhesion molecule-1 (ICAM-1) on the epithelium promotes the adhesion and migration of leukocytes to the site of infection. The aims of this study were to investigate the release of the IL-8 protein in response to Bcc infection, the impact of IL-8 on Bcc growth and intracellular survival and the expression of ICAM-1 on human lung epithelial cells and its modulation to the effect of rhlL-, LPS, or Bcc infection.

Four epithelial cell lines were used in the study; A549 cells, a human alveolar epithelial cell line, Calu-3 cells, a sub-bronchial epithelial cell line, 16HBE14o- cells and CFBE41o- cells, which are CFTR+ and CFTR- bronchial epithelial cell lines respectively. Two *B. multivorans* and two *B. cepacia* strains all induced a significant IL-8 response by 12 hrs and further increased at 24 hrs in all cell lines. Furthermore, the levels of IL-8 from Calu-3 and A549 cells were approximately three times that from 16HBE14o- or CFBE41o- cells. In two of the cell lines examined (16HBE14o- and CFBE41o-) *B. cepacia J2315*, an epidemic strain induced greater levels of IL-8 (*P* < 0.01) compared to other Bcc strains tested. The CFTR+ and CFTR- cell lines secreted similar levels of IL-8 indicating a CFTR independent induction of IL-8. However, the CFTR- cells did secrete constitutive levels of IL-8 greater than that of CFTR+ cells.

At low concentrations of rhlL- (< 10 ng/ml) growth of *B. cepacia J2315* and *B. multivorans LMG13010* was enhanced whereas at 10 ng/ml growth of both strains was significantly reduced. In contrast growth of both environmental Bcc strains remained unchanged in the presence of rhlL-. rhlL- also significantly increased the intracellular survival of both J2315 and LMG13010 in 16HBE14o- and CFBE41o- cell lines at 10 ng/ml. Although LMG13010 is more invasive in epithelial cultures, the intracellular growth of J2315 is greater than LMG13010. Furthermore, bacterial cells survive to a greater extent within the CFTR- cells than the CFTR+ cells. *ICAM-1* expression varies across different epithelial cell types with 16HBE14o- cells having the highest expression followed by CFBE41o- and Calu-3 cells and A549 cells expressing the least. LPS induces a temporary up-regulation of ICAM-1 expression by 16HBE14o- cells. In contrast, both *B. cepacia J2315* and *B. multivorans LMG13010* down regulate ICAM-1 in both 16HBE14o- and CFBE41o- cell lines. These bacterial strains were found to secrete significant levels of proteases which may play a role in the degradation of ICAM-1 molecules on the epithelial surface.

These studies demonstrate that the type of epithelial cells encountered by Bcc determines the nature and extent of the proinflammatory responses triggered. Selective and specific modulation of these responses may prevent the rapid and often fatal clinical decline associated with Bcc infection.

**Keywords:** Interleukin-8, intercellular adhesion molecule-1, cystic fibrosis transmembrane conductance regulator (CFTR), *Burkholderia cepacia* complex, proteases, human lung epithelial cells.
A novel method for Direct Cloning of large size gene or gene clusters from genomic DNA by Red/ET recombineering

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Conventional procedure to study and explore the large size gene clusters gene is time consuming and labour intensive because of library construction and screening. Recently developed Red/ET recombination or Recombineering is an ideal DNA technology to engineer and modify large size DNA molecules with no size limit and no site limit. Based on Recombineering technology, a straightforward strategy was developed to clone these gene or gene clusters from the linear genomic DNA without construction of genomic library and screening. This new DNA cloning technology, named Direct Cloning, allows cloning of up to 60kb DNA fragment from genomic DNA in one step and in two days.

Materials and Methods: Four genetically different clinical isolates of serotypes 1, 7F, 19F and 23F were studied. The former two were from invasive sites and penicillin-susceptible while the later two from respiratory sites with decreased susceptibility to penicillin. Adherence capacity of the isolates at their early-, mid- and late-log phases at 1- and 3-h exposure times to A549 cells in cell culture media and standardized at OD600 of 0.08±0.15 (~1×10^8 cfu/ml). Pneumococcal cells were then incubated at 1- and 3-h in RPMI + 2% FBS at 37°C, and growths were monitored by OD600. Growth increments from three independent experiments were determined and averaged with Kruskal-Wallist test for statistical analysis.

Results: The adherence capacity varied among the four isolates (P < 0.050) except that at mid-log phase of pneumococcal growth at 3-h exposure time (P = 0.206). Nevertheless, each isolate showed a higher adherence ability at 3-h as compared to 1-h and frequently, at both exposure times, a higher adherence was observed at mid- and late-log phases of pneumococcal growth. In similar experiments with pneumococcal cells alone in cell culture media, increases of OD600 values were observed at both post-incubation periods mainly at early- and mid-log phases. Overall, pneumococcal growth rate at 3-h incubation period was frequently twice as that at 1-h, and that at the early-log phase was frequently higher than that at the other growth phases at both post-incubation times. This indicates that the isolates were able to replicate in the cell culture media and the rate increased at a longer incubation period with the highest rate at the early-log phase. On the other hand, the adherence capacity of the isolates at their early-log phase was frequently the least. This could suggest that, although the isolates were actively replicating at this stage to provide more pneumococcal cells to adhere, they would not be in the optimum state to display their adherence ability. At mid-log phase, the replication event was generally low while that at late-log phase was barely observed except for isolate serotype 7F, which showed quite an obvious OD600 increment at both post-incubation times but at a level lower than that at the early- and mid-log phases of the isolates. In analyzing the adherence capacity at the mid- and late-log phases of the isolates, a higher adherence capacity was generally observed although the pneumococcal replication was shown to be lower at these growth stages. Discussion: These findings preliminary indicate that the adherence ability of the isolates was better at late- and late-log phases of the isolates as compared to that at the early-log phase. For the later stage, although the number of pneumococcal cells could be higher due to the higher replication rate but not all were possibly fit enough to adhere to the AS549 cells.

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Keywords: pneumococci; adherence; growth phases
A novel respiratory complex in *Desulfovibrio vulgaris* Hildenborough

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Membrane proteins are essential in vital cellular processes of all organisms including the respiratory mechanism, and they represent ca. 30% of the genomic information.

A new class of bacterial membrane oxidoreductases was found, and was named MFI (for Molybdopterin, FeS and integral membrane subunit), and then sub grouped in MFIcc for the class with four subunits, and in MFI for the group with a bigger complex with six subunits. The MFIcc class was already reported in the anoxicogenic phototropic bacterium *Chloroflexus aurantiacus* [1] and the aerobic non-phototropic bacterium *Rhodobacter sphaeroides* [2] and was suggested to be an alternative complex III. The operon for the smaller MFI complex is present in the genomes of a small group within the Deltaproteobacteria, all sulfate-reducing bacteria (SRB). This work describes the first example of a membrane bound complex from the MFI group in *Desulfovibrio vulgaris* Hildenborough. The complex is composed by three periplasmic subunits (a multihemic protein anchored to the membrane, an FeS protein and a protein that is annotated as a molybdopterin oxidoreductase like subunit) and one integral membrane protein.

Several membrane complexes have been isolated from the SRB in the last years, but there are still some points of the energy metabolism to be established, namely in the donors and receptors of the electron transfer flow across the membrane. The function of this new respiratory complex will be addressed, and since it is distinct from the MFIcc, we propose to name it as Qrc (for Quinone reduction complex) since it reduces quinones to quinols, which is the opposite function for the MFIcc. In agreement with this result the electron donors to the Qrc have to be present in the periplasm, and in fact electron transfer assays indicate hydrogenases and formate dehydrogenases as possible ones.

Expression studies also show similar results for the Qrc and other proteins directly involved in the sulfate respiratory chain, as well as a mutant in one of the Qrc subunits was shown to be unable to growth on hydrogen/formate [3].

References


Active ATPase ClpY(HslU), a homologous subunit of the eukaryotic 26S proteasome, is required to maintain replication in a nrdA101ts mutant of *Escherichia coli* at restrictive temperature

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The active enzyme ribonucleotide reductase (RNR) is a 1:1 complex of two subunits named proteins R1 and R2, each consisting of two polypeptide chains, coded by the genes *nrdA* and *nrdB*, respectively. The best-known defective RNR mutant in *E. coli* contains a thermolabile R1 subunit, coded by the *nrdA101* allele, which is inactivated in vitro after 2 min at 42ºC. In contrast, this protein is functionally active *in vivo* for 50 min at the restrictive temperature. This thermoresistance of RNR101 could be explained by the structural protection provided by the replication hyperstructure, if one accepts a model in which the RNR molecules that engage in the replication process are associated with the replication hyperstructure. Furthermore, if RNA or protein synthesis is inhibited at the restrictive temperature, the *nrdA101* mutant strain is able to replicate whole chromosomes. Since gene expression requires RNA and protein synthesis, we have examined whether the phenotype of the *nrdA101* mutant at restrictive temperature is a consequence of inhibiting de novo synthesis of a negative-acting protein.

It is established that heat shock proteases are induced after incubation at 42ºC. In this work we have conducted a time-course experiment to study the presumed heat-induced degradation of R1 and the putative rifampicin-dependent protection of R1 from degradation. Our results suggest that R1 is degraded by a protease whose synthesis might be induced either by the temperature shift and/or by the appearance of altered proteins. We tested the effect of a deficiency of certain proteases/chaperons (Lon, ClpP, ClpX, ClpB, DnaK and ClpY) on RNR101 stability and its capacity to support DNA synthesis at 42ºC. Deletion of ClpX, ClpB or DnaK, chaperones affected neither stability RNR101 nor the thermostolerance of DNA replication in *nrdA101* mutant at 42ºC. However, the stability of RNR101 is increased in the absence of Lon and ClpP proteases at 42ºC, although this effect does not correlate with an increased capacity of DNA synthesis in the *nrdA101* background at 42ºC. The results also indicate that the inhibition of the RNR degradation, observed when RNA synthesis is inhibited, is not sufficient to explain how the chromosomal replication can be completed at 42ºC.

Surprisingly, inactivation of ClpY thoroughly eliminated the capacity of DNA replication in the *nrdA101* mutant at 42ºC. ClpY is a 50 kDa ATPase that works as a component of the ClpYQ protease, which is related to the eukaryotic 26S proteasome. We discuss the role of chaperone ClpY in remodelling reactions in order to stably maintain the replication hyperstructure, in presence of an altered RNR, in *nrdA101* mutants.

Keywords Ribonucleotide reductase; protease, DNA replication, ClpY
Aerobic biodegradation of dichloromethane: new findings

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Dichloromethane (CH2Cl2, DCM) is a highly toxic volatile mutagenic and carcinogenic compound globally used as solvent, degreasing agent and intermediate for industrial synthesis. Due to its persistence and the large quantities produced industrially (about 3·10^5 tons annually worldwide), DCM has become one of the most abundant anthropogenic environmental pollutants. It was shown earlier that this toxicant can be utilized as the sole carbon and energy source by a wide range of microorganisms under both aerobic and anaerobic conditions.

Our unique collection of aerobic methylotrophic bacteria utilizing DCM as the sole carbon and energy source included 13 strains (DM1-DM13) belonging to the genus Alpha- and Betaproteobacteria, which implement serine, ribulose monophosphate (RuMP) and ribulose bisphosphate (RuBP) pathways of C_1-metabolism. Based on pheno- and genotypic characteristics, 3 new genera (Alteribacter, Methylorhabdus, Methylophilus) and 5 new species (Methylphilus helvetica, Methylorhabdus multivorum, Methylophilus leisingeri, Paracoccus methylulans, Allobacter methylphilus) could be defined. Recently, two new DCM degrading aerobic facultatively methylotrophic bacteria with the RuBP pathway for C_1-assimilation were isolated, from a garden soil sample taken near Pushchino subsequently subjected to prolonged exposure to DCM (DM15), and from contaminated soil sampled at a Volograd chemical plant (DM16). Polyphasic taxonomy studies allowed us to classify the strain DM15 as a single species of the novel genus with the proposed name "Gottschalkia methylica" gen. nov. sp. nov., and the DM16 isolate - as a new species of Ancyllobacter genus - as Ancyllobacter dichloromethanivorans sp. nov. Hence, our results clearly indicate a phylogenetic diversity of aerobic DCM degraders, and support the hypothesis that the capability for degradation of this pollutant is not limited to specific methylotrophs but develops in natural microbial populations due to the long-term selective influence of DCM.

It is known that dehalogenation of CH2Cl2 in all aerobic DCM-degrading methylotrophic bacteria characterized so far is catalyzed by cytoplasmic DCM dehalogenase (glutathione S-transferase) (DCMD) encoded by the dcmE gene. The dechlorination process results in formaldehyde used for energy generation and biosynthesis, and intracellular production of hydrochloric acid, which is excreted as H^+ and Cl^- ions to the medium. It is noteworthy, however, potential DCM-specific adaptation mechanisms for DCM transport into the cell or coping with hydrochloric acid remain to be investigated in detail.

We have shown that, for cell protection in the presence of DCM, Methyllobacterium extensus DM4, M. helvetica DM6 and A. methylovorans DM10 reduce their membrane fluidity by an increase of saturated (C16:0, C18:0) and a decrease of nonsaturated (C14:0, C18:1) fatty acids, as well as phosphatidylcholine accumulation. Further, a reduced lag phase and a faster release into the medium after DCM addition was detected for M. helvetica DM6 and A. methylovorans DM10 previously grown on minimal medium with methanol in the presence of 100mM NaCl. This effect resulted from earlier expression of the DCM dehalogenase, and perhaps also from the activation of a putative chloride exclusion system useful for DCM dechlorination process.

Also, electronic microscopy of cells of both strains DM6 and DM10, either grown with methanol in salinity stress conditions or with DCM, displayed unusual membrane-associated structures on their surface. The structures were found to contain chlorine and phosphorus atoms, and we speculate that these structures may be involved in chloride transport or the establishment of a negative charge for cell protection against high concentrations of external anions. The sensitivity of the DCM dehalogenation process to uncoupling agents (nigericin, valinomycin, carbonyl cyanide 3-chlorophenylhydrazone) revealed in several DCM degraders also suggests that the proton-motive force and/or energy-dependent processes might be involved in secondary active chloride excretion in these bacteria.

This work was funded by grant RFBR № 06-04-22000u_a-CNRS PICS № 3380, by EU Marie Curie AXIOM Project № MEST-CT-2004-08332 and National Project of Russian Federal Ministry of Education and Science № 2.1.1/6055.

Keywords: dichloromethane; dehalogenation; chloride; salinity; methylotrophy; taxonomy; physiology

Alterations by transition metals nickel, cadmium and mercury in Salmonella typhimurium growth and membrane proteins

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While some transition metals are indispensable in trace amount for most living systems, all are toxic and a serious threat to all forms of life at elevated levels. Nickel, although highly toxic, has been recognized as an essential trace element for animals, plants and bacteria; cadmium is known as toxic for all living systems; mercury is one of the most toxic elements and no beneficial function has been associated with it. Increasing levels of mercury in air and water since the beginning of the twentieth century are taking catastrophic proportions. Salmonella typhimurium, a facultative anaerobic bacterium belonging to the Enterobacteriaceae genus, is a common cause of food poisoning worldwide. Although it has been the subject of many investigations, the bacterium is still extensively studied because of its importance for human and animal health and a better knowledge of its behaviour in stressful situations is of prime importance. This investigation focuses on the effect of nickel, cadmium and mercury on S. typhimurium growth and on its membrane proteins, using cytochromes as a probe in studying the alterations caused by excess transition metals.

S. typhimurium was grown either in minimal or in enriched medium at 35°C for 24-48 h, in a rotary shaker (at 150 rpm). Each medium was supplemented with increasing concentrations of either nickel (as nickel sulphate), cadmium (as cadmium sulphate) or mercury (as mercuric chloride). Cells were harvested by centrifugation and washed twice in phosphate buffer 0.1 M, pH 7. Dithionite reduced-minus-air oxidized difference spectra of cell suspensions were recorded at room temperature (~ 22°C) using an Aminco DW2 UV/VIS spectrophotometer.

Results showed that S. typhimurium exhibited a much higher sensitivity to Hg^2+ than to the other two metal ions. After 24 h growth in minimal medium the yield decreased by 50% in the presence of 0.26 mM Ni^2+ and by 60% in the presence of either 0.008 mM Cd^2+ or 0.0008 mM Hg^2+.

After growth in enriched medium, the yield decreased by approximately 80% in the presence of 5 mM Ni^2+ (Fig. 1a) or 5 mM Cd^2+ (Fig. 1b) and by 65% in the presence of 0.03 mM Hg^2+ (Fig. 1c). In both media, for metal concentrations that led to a drastic decrease in cell yield, the cytochrome content (expressed as mmol/mg prot in the cell suspension) diminished to various extents, depending on the metal and on the cytochrome. In the presence of Ni^2+ and Cd^2+, as the metal concentration increased, the amount of cytochrome b, detectable in cells decreased more rapidly than that of other cytochromes. In the presence of increasing Hg^2+ concentrations, the amount of cytochrome d was more specifically affected.

Transition metals toxicity results from a variety of mechanisms that include, besides binding to various cellular components, reactive oxygen species (ROS) production, protein structure alterations and enzyme inactivation, essential metal ions displacement from biomolecules. The metals selected in this investigation were less prone than others to trigger ROS production and other mechanisms, such as the ability to bind to proteins and nucleic acids, probably prevailed in their toxicity. Hg^2+ in, particular, owes its extreme toxicity to its very high affinity for these groups, while Ni^2+ and Cd^2+ are known to bind to histidine and cysteine residues in proteins. Results indicated that Hg^2+ was much more toxic than Cd^2+ and Ni^2+ in S. typhimurium and affected differently the cytochromes system.

Keywords: transition metals, nickel, cadmium; mercury; Salmonella typhimurium; cytochromes
Ammonium assimilation in the eukaryotic microalgae *Chlamydomonas acidophila*

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Extremophiles are organisms that live at extremes pH, temperature, pressure, salinity or high concentration of heavy metals. They are valuable organisms to study the origin of life and for their direct application in biotechnological processes, which may involve the organisms themselves or their biomolecules. In this work, we have used the extremophile microalgae *Chlamydomonas acidophila*, isolated from the Tinto River, from Huelva (Spain), to study and characterize the enzyme glutamine synthetase, an enzyme involved in the nitrogen assimilation pathway of photosynthetic organisms.

*Chlamydomonas ac.* can live at low pH (2.5) and high metal and sulphur concentration. Nitrogen uptake, in the microalgae, is affected by the pH of the culture medium. In fact, at low pH (2.5), the uptake of nitrate is more favoured than ammonium uptake. Ammonium is considered a preferred nitrogen source for green algae and is assimilated via glutamine synthetase (GS)-glutamate synthase (GOGAT) cycle. GS transferase activity is assayed using glutamine and hydroxylamine as substrates. In *Chlamydomonas ac.*, the activity assay is dependent of Mn$^{2+}$ and AsO$_4^{3-}$. The enzyme has an optimum temperature of 40°C, and the kinetic data indicate a $K_m$ value of 27 mM for glutamine. Opposite to *Chlamydomonas reinhardii* (wild strain) *Chlamydomonas ac.* has not got GS isoenzymes, showing an only single band in native-PAGE. Other kinetic and molecular data have been also studied.

Supported by research grant nº AGL2007-65303-C02-01

Analysis of carbon source and pH-dependent transcriptional regulation of *Humicola grisea var. thermoidea* lignocellulolytic system

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Environmental pH is an important signal for fungi physiology, intervening at the transcriptional regulation of several gene products. In filamentous fungi and yeasts, the PacC zinc-finger transcription factor regulates gene expression in response to alkaline external pH. The production of enzymes involved in plant cell wall breakdown is regulated mainly at the transcriptional level. Nonetheless, the involvement of the pH-related regulatory pathway in the lignocellulolytic enzymes expression has not been extensively studied. We have demonstrated that the thermophilic deuteromycete *Humicola grisea var. thermoidea* is a potent cellulases producer, presenting a considerable potential for agricultural wastes bioconversion processes. Results of our group support the existence of a pH regulatory pathway for *H. grisea var. thermoidea* transcriptional regulation. In this work, we have performed by quantitative real time RT-PCR a time course trancriptional analysis of several *H. grisea* genes. Eight lignocellulolytic genes (cbhl1.1, cbhl1.2, egf1, egf2, egf3, egf4, bgld and syn1) and two transcription factors (pacC and creA) were analyzed in the presence of simple (glucose) or complex (sugarcane bagasse) carbon sources and in acid or alkaline medium conditions. The qRT-PCR analysis revealed an early and strong induction of transcription of almost all lignocellulolytic genes, in a synergistic way, when the mycelia were grown with the complex carbon source and in alkaline conditions (pH 8.0). The only exception was egf4, that was acid induced. An opposite pattern of expression of the two transcription factors was observed. While pacC was induced in alkaline conditions and strongly repressed in presence of glucose, creA was induced by glucose and repressed in alkaline conditions. By electromophoretic mobility shift assays (EMSA) with upstream regulatory sequences of pacC, we showed that exists an in vitro interaction between the proteins PacC and CreA with pacC upstream regulatory sequence, where the both factor compete by the same binding site. Taken together, this data corroborates our previous evidences supporting the existence of a pH regulatory pathway for *H. grisea* transcriptional regulation by PacC. Moreover, PacC is probably transcriptionally regulated by itself and may suffer influence of the carbon repression mechanism mediated by CreA.

**Key words:** pH, transcriptional regulation, PacC, CreA
Assaying the Single and Combined Genotoxicity of *Calotropis procera* Ait Latex and Chlорcyrin in *Aspergillus terreus*.

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The conidial spores of *Aspergillus terreus* were treated singly and in a combination (alternatively and simultaneously) with latex of *Calotropis procera* Ait (used as natural insecticide) and the synthetic insecticide Chlорcyrin. The single mutagenic effects of *Calotropis procera* Ait latex and the insecticide Chlорcyrin were tested by treating conidia of *Aspergillus terreus* with four different concentrations of *Calotropis procera* Ait latex: 5%, 10%, 20% and 40%. And with five concentrations of Chlорcyrin; 0.2ml/L, 0.4ml/L, 0.6ml/L, 0.8ml/L and 1ml/L (the field concentration) for different exposure times individually. Whereas, the combined mutagenic effects of the both substances, were tested in three ways of treatments. First, the conidia of *Aspergillus terreus* were exposed to the optimal dose of *Calotropis procera* Ait latex (20%) for 45min., followed by exposure to the optimal concentration of Chlорcyrin (0.8ml/L) for 45 minutes. Second, same conidia were exposed to Chlорcyrin followed by *Calotropis procera* Ait latex with the mentioned concentrations and exposure times. Finally, third treatment included the exposure of the fungus conidia to a mixture of the two substances (same concentrations) for 90 minutes.

As a result, it was found in the first single treatment that an increase of *Calotropis procera* Ait latex concentration and exposure time led to an increase in auxotrophic mutants percentage, and the optimal dose for inducing mutation was found to be (20%) for 45min. of exposure which induced a percentage of 1.6 auxotrophic mutants. Similar trend were obtained in the second single treatment, that an increase of Chlорcyrin concentration and exposure time led to an increase in auxotrophic mutants percentage, and the optimal dose was found to be 0.8ml/L for 45min. of exposure (the concentration of which 3.6% of auxotrophic mutants were obtained). Thus percentage of auxotrophic mutants induced by Chlорcyrin exceeded noticeably that of *Calotropis procera* Ait latex. Furthermore, Chlорcyrin shows to be a potent mutagenic substance as compared with *Calotropis procera* Ait latex which shows a milder mutagenic effect, and more cytotoxic effect. All combined treatments revealed an antagonistic effect.

Availability of CO$_2$ concentrating mechanism in extremely haloalkaliphilic cyanobacteria 'Euthalothecia natronophila' from soda lake Magadi (Kenya)

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'Euthalothecia natronophila' is obligate extremely haloalkaliphilic unicellular cyanobacteria, isolated from soda lake Magadi (Kenya). It develops optimally in concentrated Na$_2$CO$_3$+NaHCO$_3$ brines (1.7 M Na$_2$CO$_3$+NaHCO$_3$, 1.2-2.4 M Na$^+$, pH 10-10.5 in laboratory conditions). Thereby it is adapted to existence in drying up brines right to beginning of evaporites precipitation. This feature may cause development of cyanobacteria and their active functioning as primary producers during dry seasons in natural conditions.

Extremely haloalkaliphilic microorganisms are under special interest nowadays because of their unique characteristics due to environmental conditions. But most of the investigations dealing with CO$_2$ concentrating mechanism (CCM) in cyanobacteria use model freshwater and marine strains, such as *Synechococcus* PCC7942, *Synechocystis* PCC6803 and *Synechococcus* PCC7002. Our aim was to investigate availability or absence of active CCM in 'E. natronophila' and its role as adaptive strategy for existence in Na$_2$CO$_3$+NaHCO$_3$ brines.

As a result we revealed all CCM components in 'E. natronophila' cells (Fig. 1): transport systems (TS) for inorganic carbon (C$_i$), carboxysomes (CS) and carbon anhydrases (CA). Their quantity and functioning depends on the concentration of CO$_2$/HCO$_3$ in medium. Transport of C$_i$ into the cells provides with necessary amount of C$_i$ in different cultural conditions. It means that their role is not only for C$_i$ concentrating, but also for preventing C$_i$ surplus in 'E. natronophila' cells what is necessary in soda environments for intracellular pH-homeostasis as C$_i$/HCO$_3$/CO$_2$ are buffer components.

Presence of carbon anhydrases of ß-type (ß-CA) was shown by Western blot with antibodies to cytosolic ß-CA from *Coccomyxa* sp. Its amount in cells increases with a decrease of C$_i$ concentration in cultural medium as well as quantity of carboxysomes, established by transmission electronic microscope. We didn’t find extracellular ß-CA in 'E. natronophila' cells using antibodies to thylakoidal ß-CA (anti-Caß) of *Chlamidonas reinhardtii*. Also trustworthy CA activity of intact cells was not registered. We consider it the absence of extracellular CA in 'E. natronophila'. But it was found earlier (Kupriyanova et al., 2007) in glycoalyx of alkaliphilic strains of *Microcoleus chthonoplastes* and *Rhodobacter* lineare. So the role and importance of extracellular CA have to be discussed.

Thereby, extremely haloalkaliphilic cyanobacteria 'Euthalothecia natronophila' possess CCM which is similar in structure to CCM of model nonextremophile strains of cyanobacteria, but it differs in some properties. Especially it is in affinity of TS which is three degree less than in nonextremophile cyanobacteria. The role of CCM components in haloalkaliphilic cyanobacteria may be suggested not only as “concentrating”, but more exactly as “regulative”.

This work is financially supported by RFBR №08-04-00804-a, Presidium of RAS Program (‘Biosphere origin and evolution’) and Contract with Rosnauka № 02.512.12.0027. The authors are also very grateful to Е. Kupriyanova, А. Markelova and N. Pronina (Institute of Plant Physiology RAS, Moscow, Russia) for their kind help in this investigation and discussion of results.

Keywords: extremely alkaliphilic, 'Euthalothecia natronophila', CO$_2$ concentrating mechanism (CCM), C$_i$ transporters, carboxysomes, carbon anhydrases.
Berberine vs. *P. polychaetum* alkaloid extract for antimicrobial activity

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The emergence of widespread bacterial drug resistance is a major threat to public health. Natural products, specifically plant extracts, serve as invaluable sources for the development of novel anti-microbials. Berberine, the major alkaloid in the alkaloid extract of the endemic plant *Papaver polychaetum* has a long history of medicinal use. It has demonstrated significant antimicrobial activity against different organisms including fungi and is relatively nontoxic to humans. In this study, the antimicrobial effect of the alkaloid extract of *P. polychaetum* and pure berberine against *E. coli* K12 has been investigated in order to determine the synergetic or additional effects of the compounds in the plant extract. Following determination of the minimum inhibitory concentration of berberine as 1250 μg/mL, *E. coli* cells were grown in the presence of 750 μg/mL berberine or *P. polychaetum* alkaloid extract. Inhibition of growth and protein expression differences upon addition of the pure alkaloid or the alkaloid extract of the plant extract were compared against the control group.

Biotechnology Pseudomonas strain early defence gene expression correlates to pathogenic potential

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The genus *Pseudomonas* contains taxa that are of biotechnological interest and have been used commercially in applications ranging from biocontrol to bioremediation. *Pseudomonads* are common constituents of soil and water environments as well as plant and animal hosts. Nevertheless, some strains may be pathogenic to humans such as *P. aeruginosa* (Pa) that infects the lungs, urinary tract and wounds of immunocompromised individuals and is a major cause of hospital acquired infections. Therefore, *Pseudomonads* vary with respect to impacts on immune and cell recognition gene expression.

The goal of this study was to determine differences in early defence gene expression in mouse macrophage cells (J774A.1), that were exposed to *Pseudomonas* strains, used in Canada (Pa, *P. fluorescens* (Pf) and *P. stutzeri* (Ps)). Real time PCR was used to profile the transcript abundance of toll-like receptors, inflammatory response and housekeeping genes at 200 minutes. The profiles for each strain and magnitude of significant gene expression changes were ranked by strain and correlated to bioreduction and cytotoxicity assay results from 24 hr exposures.

All strains caused significant changes in proinflammatory genes (Tnf, IL6, IL1b), toll like receptors (Tlr2 and Tlr6) and genes in the NFkB Pathway. However, changes in gene expression in response to Pa strains were either unique, or greater in magnitude than for tested Pf and Ps strains. These results are consistent with greater cytotoxicity and pathogenic potential of Pa strains observed in other in vitro studies at later time points. Taken together, defence gene expression profiling has the potential to provide an early indication of cell perturbation, relative to other in vitro assays, for safety testing of microbes used in biotechnological applications.

**Keywords:** Pseudomonas, biotechnology, biosafety
Cadmium effect on KlHIS4 promoter DNA binding factors

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The analysis of KLHIS4 promoter sequence showed the presence of three sites similar the Gcn4/Yap1 binding sites. Yap1 is a transcriptional factor involved in the heavy metal transcriptional regulation, so we have analysed the implication of these three sites in cadmium transcriptional regulation. A specific band, Cd2, was characterised in the region encompassing positions -283 to -276 (Fragment 2), which disappeared in the presence of cadmium or when the Gcn4/Yap1 consensus was mutated. The binding of a second factor causing the Cd3 band is also dependent on the Gcn4/Yap1 consensus at position -283 to -276, but does not respond to cadmium. The gel-shift pattern of region -384 to -323 (Fragment 4) with no consensus was also altered by cadmium, thus, the cadmium transcriptional response of KLHIS4 appears to be modulated by multiple promoter elements.

Keywords: yeast, Kluyveromyces, HIS4, cadmium, biosensor.

Characterisation of the NrfH cytochrome c quinol dehydrogenase from Desulfovibrio vulgaris

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The NrfH cytochrome c is a membrane-anchored tetraheme cytochrome c that oxidises the quinone pool and is the physiological partner of the NrfA nitrite reductase. NrfH belongs to a wide family of cytochrome c quinol dehydrogenases that play a crucial role in bacterial respiration, by oxidizing menaquinol and transferring electrons to various periplasmic oxidoreductases [1,2]. These cytochromes are very widespread in bacterial respiratory chains. The NrfHA proteins form a bacterial membrane bound complex that reduces nitrite by the oxidation of menaquinol [1,2].

We have determined the structure of the native NrfHA complex [2], and elucidated its menaquinol binding site through the structure of the NrfHA bound to 2-heptyl-4-hydroxyquinoline-N-oxide (HQNO), which is shown to act as a competitive inhibitor of the NrfH quinol oxidation activity [3]. The menaquinol-binding site is close to heme 1 of NrfH, which has very unusual heme coordination. Here we present further studies of the characterization of the NrfH proteins from two different organisms, Desulfovibrio vulgaris a sulfate-reducing Deltaproteobacterium and Wolinella succinogenes a nitrite-reducing Epsilonproteobacterium.

Characterization of KlTup1 repressor.

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The general repression complex containing the proteins Tup1p and Sun6p is a conserved global regulator of transcription present in different organisms from baker’s yeast through mammal, and serves as a model for the study of similar co-repressor complexes in higher eukaryotes. Tup1p forms a complex in vivo with Sun6p, and regulates a wide variety of gene families or regulons (genes regulated by cell type, glucose, oxygen, DNA damage, and other signals) through its recruitment to target genes by association with regulon-specific DNA binding proteins through the RNA transcriptional machinery (1). Also this complex mediated repression involves interactions among the corepressor and hypoacetylated histones, histone deacetylases, where nucleosome positioning is part of this process, in a subset of these regulated genes, showing the importance of chromatin modification states in Tup1p-Sun6p mediated repression (2). To investigate the evolutionary conservation of these functions, the KlTUP1 gene from Kluyveromyces lactis has been cloned, by complementation of a Saccharomyces cerevisiae tgt1 null mutation. The nucleotide sequence predicts a KlTup1p consists of 682 amino acids with a 62% identity to ScTup1p. A comparative analysis of transcriptional regulation of KITUP1 in different carbon sources medium has been carried out. Here, we report a structural and functional analysis of KlTup1 repressor.

Keywords: yeast, transcriptional regulation, amino acid pathway, Kluyveromyces lactis.

References:

Acknowledgements: Supported by grant PGIDIT06XIB103086PR from Xunta de Galicia (Spain).

Construction of a plasmid vector for thermoacidophilic crenarchaeon Sulfolobus acidocaldarius

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Recent studies regarding thermophilic archaea have provided many experimental data for better understanding of these interesting microorganisms. In addition, complete genomic sequences show the structures and organization of genes, suggest certain metabolic pathways, and provide comparisons at the genome level with other organisms. However, the sequence data also generate many new questions which require more powerful genetic tools to study. A host-vector system is a particularly useful tool for molecular biological and genetic studies (i.e. cloning, expression, complementation, promoter analysis, interference of genes), and may help to answer various questions that arise from current study of thermophilic archaea. The extreme thermoacidophiles of the genus Sulfolobus are among the best-studied archaebacteria but have lacked small, reliable plasmid vectors. Here we report the successful construction of a series of Sulfolobus-Escherichia coli shuttle vectors. Members of Sulfolobus are thermoacidophilic crenarchaotes that can be cultivated easily under aerobic and heterotrophic conditions in both liquid and solid media. As a result, they have been considered good model organisms for genetics of thermophilic archaea.

A host-vector system for Sulfolobus acidocaldarius based on the pyrEF complementation was developed. This consisted of pyrimidine auxotrophic strains and a plasmid vector, designated pSAV1. The pSAV1 vector consists of the complete pRN1 plasmid of S. islandicus, the ColEl origin and beta-lactamase gene derived from E. coli cloning vectors, and the pyrEF operon of S. solfataricus. Purified plasmid in which the GCCC sites were methylated was used for transformation of pyrimidine auxotrophic strains of S. acidocaldarius. The transformants were selected directly on a xylene-tryptone (XT) solid medium without any prior liquid culturing, and grew as well as the wild type in uracil-free medium. After replication in S. acidocaldarius, pSAV1 was successfully recovered from cultures of transformants by the standard alkaline lysis method and could be used for transformation without further amplification or methylation. The yield of plasmid from the culture increased after exponential phase and reached about 20 ng/mL culture in the stationary phase. This suggests that the copy number of pSAV1 in a host Sulfolobus cell was at least 14, probably about 20, in the stationary phase.

To determine the stability of the pSAV1 in S. acidocaldarius cells, the transformant (S. acidocaldarius Pyr [pSAV1]) was grown in XT medium without uracil and incubated at 75°C. After several times of sub-cultivations, the plasmid DNA was extracted and digested with restriction enzymes, and analyzed by agarose gel electrophoresis. The restriction endonuclease cleavage patterns of the plasmid DNA showed no difference with the patterns of the original pSAV1, indicating that there is no obvious deletion or rearrangement in the plasmid. The pSAV1 seems very stable and good potential to be initial plasmid to develop cloning or expression vectors of S. acidocaldarius.

Keywords: plasmid vector, Sulfolobus, pSAV1, pRN1
Construction of auxotrophic mutants of *Methylophilus methylotrophus* AS1 by recombination-mediated marker exchange between linear DNA and bacterial chromosomes in cells carrying the *Escherichia coli* aroP transporter gene: application for Phe production from methanol

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The non-pathogenic, Gram-negative bacterium *Methylophilus methylotrophus* AS1 is able to grow efficiently on methanol, methylamine or trimethylamine as a carbon source, and it uses the ribulose monophosphate pathway for fixation of formaldehyde produced by the oxidation of these compounds. The aim of this investigation was to generate strains of *M. methylotrophus* AS1 with a potential for the industrial production of aromatic amino acids (AroAAs) from methanol. As a rule, investigations of species-specific features of the metabolic pathway and construction of auxotrophic mutants are a prerequisite for future breeding of industrial strain-producers. Auxotrophic mutants have been difficult to isolate from obligate methylotrophs using standard methods. *M. methylotrophus* AS1 could grow in the presence of high concentrations of analogs of AroAAs and some amino acids (Val, in particular) that significantly exceed the inhibitory values for other Gram-negative bacteria. These results indirectly confirmed suggestions that the failure to isolate methylotrophic auxotrophs resulted from the inability of amino acids to permeate the cytoplasmic membrane.

In the present study, a new construction method for aromatic-auxotrophic mutants is described for *Methylophilus methylotrophus* AS1. It is based on the preliminary Mu-driven integration of the *Escherichia coli* aroP gene, which encodes the common aromatic amino acid transporter, in the genome of *M. methylotrophus*. The resulting recombinant strain, which has increased specific permeability to certain amino acids and their analogs, was used for mutagenesis. Mutagenesis was carried out by recombination-mediated substitution of the target genes in the chromosome by linear DNA using the FLP-excisable marker targeted by cloned homologous arms greater than 1,080 bp in length. *M. methylotrophus* AS1 trpE, tyrA, phyC and aroG genes were cloned in *E. coli*, sequenced, disrupted in vitro using a Km-marker and electroporated into an aroP-carrier recipient strain. This approach led to the construction of a set of auxotrophic marker-less methylotrophic mutants that had target genes in the chromosome destroyed.

The obtained mutants and developed method of Mu-driven integration of recombinant DNA in the *M. methylotrophus* AS1 chromosome were used for construction of strains producing L-phenylalanine from methanol (U.S. patent 63505966)

**Keywords** methylotroph, Mu-driven integration, marker-less mutants

Deficient activity of DnaA protein allows proficient replication at restrictive conditions for ribonucleotide reductase mutants

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Ribonucleotide reductase (RNR) is the only enzyme required specifically under aerobic growth for the formation of deoxyribonucleotides in *E. coli*. The best-known defective RNR mutant in *E. coli* contains a thermolabile R1 subunit coded by the *nrdA101* allele. This mutated enzyme is inactivated in 2 min in vitro, but, in vivo, a thermoresistant period of 50 min has been observed in the *nrdA101* mutant strain. The observed thermoresistant period of the thermosensitive *nrdA101* mutant strain in vitro is further extended by inhibiting RNA synthesis, allowing cells to complete replication at 42°C. RNA and protein syntheses are required to initiate chromosome replication. To examine whether the inhibition of new initiation events was related to the extensive thermoresistant period, we studied this phenotype in double *nrdA101* dnaA mutants.

In this work we show an increased thermoresistant period of chromosomal DNA replication in the *nrdA101* strain when the DnaA protein is deficient or inactivated, or when RNA or protein synthesis is inhibited. By using flow cytometry and marker frequency analysis, we found that the presence of dnaB deletion alleles allowed the *nrdA101* strain to replicate the whole chromosome at the restrictive temperature. Furthermore, we show that the double dnaB174 *nrdA101* mutant strain is not only able to complete ongoing replication rounds but also is able to initiate new rounds of chromosomal replication at the restrictive temperature. Growth of strain *nrdA101* at the restrictive temperature causes filamentation and affects the DNA distribution throughout the cell. However, phase contrast microscopy observation of the *nrdA101* dnaA double mutant strains growing at the restrictive temperature showed no filamentation. Nucleoid segregation was studied by fluorescence microscopy of DAPI stained cells that had been grown for 4 hours at the restrictive temperature in the presence of cephalaxin, to inhibit cell division. These cells showed a normal nucleoid segregation in the *nrdA101* dnaA double mutants, in contrast with the abnormal segregations observed in the *nrdA101* strain. The aberrant division pattern and nucleoid segregation observed in the *nrdA101* strain have been attributed to uncoupling of replication and cell division. Our results are consistent with this notion, as they show that impaired replication, cell filamentation, and alteration of nucleoid segregation observed in the *nrdA101* strain at 42°C were eliminated when DnaA protein activity is deficient.

Our results suggest that the long thermoresistant period of replication in the *nrdA101* mutant strain at the restrictive temperature could be ascribed to a reduction in the number of replication cycles, either induced by inhibition of RNA/protein synthesis or by a deficiency of DnaA protein. Given that replication fork progression is impaired in the *nrdA101* mutant, we propose that a reduction in the number of forks along the chromosome could increase the replication capacity of the mutant under restrictive conditions.

**Keywords** Ribonucleotide reductase, *nrdA101*, dnaA, DNA replication
Degradation of the Compatible Solute Ectoine

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For maintenance of osmotic equilibrium at elevated salt concentrations halophilic Bacteria synthesize and/or accumulate organic osmolytes. In response to the osmotic stress these molecules can reach high cytoplasmic levels without disturbing the cell’s metabolism and are, therefore, named compatible solutes.

One of the predominant compatible solutes in halophilic Bacteria is the aspartate derivative ectoine. For ectoine it was shown that it possesses protective properties in stabilizing enzymes and even whole cells against stresses such as UV radiation or cytotoxins [1]. Recent studies show that ectoine also protects against particle-induced inflammation in lung epithelia [2] and small bowel from ischemia and reperfusion injury [3]. Its protective properties make ectoine a valuable compound which is marketed in health care and skin care products. Ectoine is therefore produced annually on the scale of tons by industry in a biotechnological process [4] with the halophilic γ-proteobacterium Halomonas elongata used as producer strain. It is therefore of great interest to elucidate the degradation pathway of ectoine in this organism.

Inspection of the genome of H. elongata helped to identify two gene clusters named doeABX and doeCD (degradation of ectoine) that are involved in ectoine degradation. The actual enzymes responsible for ectoine degradation are encoded by doeA, doeB and doeD. Recombinant expression of doeA revealed that the DoeA protein catalyzes the hydrolysis of ectoine to N-acetyl-diaminobutyric acid (N-Ac-DABA). N-Ac-DABA serves as substrate for the subsequent deacetylation reaction by DoeB. Based on further genetic and biochemical analyses we propose that the pathway proceeds from diaminobutyric acid to aspartate via aspartate semialdehyde catalyzed by newly discovered transaminase DoeD and the dehydrogenase DoeC, respectively.

RT-PCR experiments showed that doeAB is transcribed together along with a third ORF named doeX. The transcriptional initiation site of the doeABX operon was mapped by rapid amplifying of cDNA ends (RACE). Inspection of the DNA sequence upstream of the initiation site revealed the presence of a putative –10 and –35 sequences that resembles the consensus sequence of sigma 70 dependent promoters. The newly identified doeX locus is coding for a putative protein with a calculated molecular mass of 17.9 kDa. The deduced amino acid sequence of DoeX shows a high degree of identity to transcriptional regulator proteins of the AsnC/Lrp family. Electrophoretic mobility shift assays (EMSA) proved that DoeX is indeed a DNA-binding protein with significant binding affinity to the promoter region of doeABX.

Detection and genetic characterization of vanA-containing Enterococcus strains in healthy Lusitano horses

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Running title: vanA-containing Enterococcus in Lusitano horses

Ninety faecal samples from Lusitano horses were investigated in order to detect the presence of vancomycin-resistant enterococci. vanA-containing enterococcal strains were detected in 4.4% of these faecal samples and one isolate per sample was characterized (one E. faecium and three E. faecalis isolates). vanA isolates showed high level vancomycin (MIC ≥128 mg/L) and teicoplanin resistance (MIC 64 mg/L), as well as resistance to ciprofloxacin, erythromycin and tetracycline. The tet(L) and erm(B) genes, related with tetracycline and erythromycin resistance, respectively, were found in all vanA isolates. Enterococcal strains with intrinsic vancomycin resistance (vanC-L) were found in four faecal samples. The intestinal tract of Lusitano horses can be a potential reservoir for vanA-containing enterococci.

Keywords: vanA, enterococci, Lusitano horses, Portugal

References:
Differences in vitamin requirements for *Streptococcus pneumoniae* serotype 14 cultivation in flask and in reactor

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Introduction: *Streptococcus pneumoniae* is a major cause of mortality in developing countries where more than 1 million of people die every year. The emerging of multiresistant strains is a main obstacle in the treatment of pneumococcal disease. Faced with these facts, the vaccination is the best solution to reduce the mortality and morbidity rates. Currently, the available vaccines are based on the capsular polysaccharide (PS) protection. The PS is the most important pneumococcal virulence factor and the immunogenicity of the different pneumococcal PS is the base for *S. pneumoniae* classification in serotypes. These serotypes have an irregular regional and age group distribution, which should be taken into account in vaccine design. In Brazil, the serotype 14 is the most common serotype and often affects young children. Despite the importance of pneumococcal antigen production, very little attention has been devoted to pneumococcal nutritional requirements and metabolism. Pneumococcus is an aerobics fermentative microorganism and produces mainly lactate. The vitamin metabolism, on the other hand, varies among strains and the vitamin requirements are influenced by the composition of medium utilized.

Objectives: Determine vitamin requirements of *S. pneumoniae* serotype 14 in chemically defined medium, in order to reduce the number of compounds used and the PS production cost, consequently.

Materials and Methods: The *S. pneumoniae* serotype 14 strain ST 5287/09 was used in all experiments described. The stock culture was prepared in Todd-Hewitt (Difco) supplemented with 0.5% yeast extract (Difco) + 40% glycerol and stored in liquid nitrogen. The inocula were prepared from the stock culture in 50 mL of complete medium (a variant of the Hoeprich medium, containing glucose, acid-hydrolyzed casein, yeast extract, L-glutamine, asparagine, choline, thiglycolic acid and salts) in atmosphere of ~3% CO₂, 37°C, under static cultivation. When these cultures achieved the exponential phase, the inocula were centrifuged, washed with NaCl 0.85% and transferred to flasks with 50 mL of Chemically Defined Medium - CDM (van de Rijn & Kessler, 1980), in order to get an initial optical density at 600 nm (OD₆₀₀) around 0.1. The growth profile was evaluated in flask with complete CDM (control) and CDM without each vitamin/cofactor to be tested: riboflavin, nicotinamide, pantothentic acid, thiamine, p-aminobenzoic acid, biotin, folic acid, pyridoxamine, pyridoxal, p-NAD, pyridoxamine + pyridoxal and p-aminobenzoic acid + folic acid. New cultures were performed in flask and reactor only with the indispensable vitamins identified in the previous step. For reactor cultivation, the inoculum preparation was the same as described above and all fermentations started with OD₆₀₀=0.1. Batch fermentations were conducted in 1.5L-bioreactor BioFlo 3000 (New Brunswick Scientific Inc.), under nitrogen atmosphere (0.5L/min), at 37°C and 100 rpm; pH was controlled at 7.0 by addition of 5M of NaOH. Polypyrrolane was used as antifoam agent. The bioreactor parameters were monitored by the BioCommand software (New Brunswick Scientific Inc.) version 2.5.

Results and Discussion: In flask experiments, only the presence of the vitamins nicotinamide and pantothenic acid affected the cell growth in comparison with control. The complete CDM achieved a maximum OD (OD_max) of 2.5 and a maximum specific growth rate (μ_max) of 0.6 h⁻¹. The individual omission of nicotinamide and pantothentic acid decrease the cell yield in 25% and 73%, respectively, while the same OD₆₀₀ and μ_max of the control was observed when both pantothenic acid and nicotinamide were added. However, this behavior was not observed in reactor cultivation. The complete CDM yielded OD₆₀₀=4.0 and μ_max=0.78 h⁻¹, whilst for CDM with nicotinamide and pantothenic acid only these values were, correspondingly, 0.9 and 0.5. Even when the inoculum was prepared using the same medium of the reactor (CDM with nicotinamide and pantothenic acid only) to avoid cell death during centrifugation, the same behavior was observed. A little increase in cell growth was achieved (OD_max~1.73) when a pulse of the omitted vitamins was given at the end of exponential phase. This suggests that one or more of those omitted vitamins were indispensable for cell growth in reactor cultivation.

Conclusions: The vitamin requirements were different in flask and reactor cultivation, affecting the growth performance. This behavior is undesirable, as long as the PS production is associated to cell yield. Given the optimal conditions to stimulate cell growth in reactor, as pH control and homogeneity, it is hypothesized that the bacteria did not have time to adapt to the imposed vitamin limitation, since no lag phase was observed. On the opposite, it was observed 2 hours of lag phase in all flask cultivations. Futures experiments will be performed to identify which vitamins should be stimulated to increase the cell growth in reactor cultivation and obtain a similar yield observed using complete CDM.

Financial support: FAPESP, CAPES.

Dissection of the *Escherichia coli* rpsB promoter: in vivo analysis of activity and stringent response.

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Expression of the *Escherichia coli* rpsB-tsf operon encoding essential components of translational machinery, ribosomal protein S2 and elongation factor Ts, is driven by a single promoter which is highly conserved among γ-proteobacteria. The rpsB promoter (P_rpsB) bears a rare combination of specific features: it belongs to the extended minus 10 promoter class and harbors a TGTGg-extension upstream of the TATAAA hexamer, a suboptimal -35 region (TTGGTG), a GC-rich discriminator GCCGCG, and it uses CTP as the initiating nucleotide. The 5'-TGTG-extension is known to stabilize the open complex of RNA polymerase with the promoter, while the GC-rich discriminator is considered as a destabilizing factor, in particular during stringent response. Thus, the rpsB promoter comprises two elements which in theory should have opposite impact on promoter activity under certain conditions. In this work, we have examined the impact of site-directed mutations in individual DNA elements within P_rpsB on expression of a single-copy lacZ gene and determined whether P_rpsB is controlled by ppGpp during amino acid starvation. The results show that the promoter activity largely depends on 5'-extension TGTG, and the loss of activity upon alteration of this sequence cannot be compensated by introducing a consensus -35 region. Furthermore, we have shown that the promoter activity is negatively regulated by ppGpp in vivo, and the mutations converting the GC-rich discriminator into the AT-rich sequence abolished the negative stringent response. The data obtained confirm the position of the rpsB promoter previously defined by mapping the 5'-ends of in vivo rpsB transcripts and demonstrate that the extended -10 promoter may be subjected to negative stringent control due to the presence of a GC-rich discriminator.

This work is supported by RFBR grant 09-04-01014

Keywords rpsB-tsf operon; extended -10 promoter; site-directed mutagenesis; stringent response.
Diversification of nucleoid-associated proteins in bacteria revealed by comparative proteomic analysis

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Bacterial nucleoids are composed of hundreds of proteins including nucleoid proteins like Hu and H-NS, but most of the proteins have not been identified yet. In this study, we isolated nucleoids of Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis and Staphylococcus aureus from log phase cultures, and identified their associated proteins by LC-MS/MS. As the nucleoid-fraction specific proteins, 228, 102, 120 and 59 proteins were identified from E. coli, P. aeruginosa, B. subtilis and S. aureus, respectively. Comparative genomic analyses with this set of proteins showed that only Hu is common among them, indicating that bacteria have varied the nucleoid-associated proteins. The variety of the identified proteins suggests that bacteria have evolved different strategies to adopt environment stresses.

Keywords: Nucleoid; LC-MS/MS; Comparative genomics

Effect of cyanide and azide on growth and membrane proteins in Salmonella typhimurium

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Potassium cyanide and sodium azide, powerful poisons that block respiration, are constantly released in the environment because of their extensive industrial use. A number of plants and microorganisms are equipped with cyanide-insensitive respiration and some scavenge and/or metabolize cyanide. Salmonella typhimurium, a gram-negative, facultative anaerobic enterobacterium, is a major cause of disease in human and animals, and frequent outbreaks of salmonellosis are a great concern in food industry. S. typhimurium respiratory chain includes cytochrome d, a terminal oxidase reputedly relatively insensitive to cyanide; this would give the bacterium an advantage over other species in an increasingly contaminated environment. The control of S. typhimurium population being of great importance from a health point of view as well as from an economical point of view, a better knowledge of the bacterium resistance to toxic compounds is needed. In this research the effect of millimolar concentrations of cyanide and azide on S. typhimurium growth and on its membrane proteins, using cytochromes as a probe, was investigated.

Results showed that S. typhimurium was able to sustain growth in the presence of millimolar concentrations of either potassium cyanide or sodium azide and that it was more resistant to sodium azide than to potassium cyanide. As shown in Fig. 1, the yield after 24 h culture was 74% in the presence 5 mM sodium azide (Fig. 1a) compared to 28% in 5 mM potassium cyanide (Fig. 1b), and 24% of the control in 10 mM azide (Fig. 1a) compared to 0% in 10 mM cyanide (Fig. 1b). As revealed by their reduced-minus-oxidized difference absorption spectra, cells grown in 3 mM azide exhibited the same cytochromes content as the control, namely cytochromes b560, b562, and d, while cells grown in 5 mM cyanide exhibited an altered cytochromes pattern.

When cyanide was added to a suspension of oxidized S. typhimurium cells, a cytochrome d-CN complex was formed, detectable by difference spectrophotometry, while no complex was detectable when azide replaced cyanide. However, when both compounds were added to the cells, azide competitively inhibited the cytochrome d-CN complex formation. Secondary and Dixon plots were parabolic, suggesting that two azide molecules bound to cytochrome d. Data also showed that cyanide had more affinity than azide for cytochrome d in S. typhimurium.

Although binding of cyanide or azide to cytochrome d, and the ensuing inhibition of the cytochrome has been the subject of considerable research spanning many decades, important aspects of this inhibition still remain unclear. The inhibitors competition reported here is bringing some additional information about cyanide and azide binding to cytochrome d in S. typhimurium. Binding of azide which could not be directly detected under our experimental conditions, was evidenced by studying the kinetics of cyanide binding to cytochrome d in the presence of azide. Results showed that azide competed with cyanide for the same binding site. Finally, although cyanide and azide did bind to cytochrome d in S. typhimurium, the bacterium was able to grow in the presence of millimolar concentrations of both compounds, thus exhibiting a resistance conferring it an advantage in hostile environment.

Keywords: cyanide; azide; toxic waste; bioremediation; Salmonella typhimurium; cytochromes d

![Figure 1](image-url)
Enhancement of extracellular purine nucleoside and AICA ribonucleoside accumulation by Bacillus strains through the genetic modification of genes involved in nucleoside export

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Bacillus subtilis and its close relatives have been developed and engineered as industrial producers of primary metabolites, including vitamins (riboflavin, folic acid and biotin) and purine nucleosides (inosine and guanosine) as precursors of flavor-enhancing nucleotides.

We have previously shown that overexpression of genes encoding amino acid efflux transporters markedly improved the productivity of amino acid-producing strains (Zakataeva et al., 1998; Livshits et al., 2003). We have also found that the product of the Escherichia coli nepl (yicM) gene and its homologue (YidB) are involved in export of purine nucleosides in B. subtilis and B. amyloliquefaciens (Grunskiy et al., 2005; Zakataeva et al., 2007). It could be predicted that enhancement of the expression of these genes might also increase purine nucleoside accumulation by the respective producing strains. To study the role of modified expression of the phuE and yicM genes on extracellular accumulation of purine derivatives by B. amyloliquefaciens-producing strains, several genetic modifications were introduced into the chromosomes of these naturally non-transformable bacteria by the replacement recombination method.

This method has been engineered to introduce marker-free deletions, insertions or point mutations into the chromosome of Bacillus strains, including those that are naturally non-transformable. The method is based on the cooperative effect of an homologous transfer and replacement recombination process, which occurs at a very high frequency due to the use of a thermostable rolling-circle replication plasmid as a delivery vector, and also colony PCR analysis for screening. Use of PCR primers with mismatches at the 5' end enables the selection of strains containing a single point mutation in the target gene. This method is efficient, fast and allows the generation of any of these genetic modifications without positive selection, the use of a counter-selectable marker or a special prerequisite strain. These features are especially important for metabolic engineering of food-grade industrial strains.

Using this method, the phuE gene was overexpressed from the chromosomal of the insine and guanosine producer AJ1991 and the AICA (aminimidazol carboxamide) ribonucleoside producer AJ1991 iapBR.

Overexpression was achieved through chromosomal integration of the phuE::cm mutation in the 5' untranslated region of phuE, which is known to significantly enhance the gene expression level. Moreover, E. coli nepl was expressed under control of the B. amyloliquefaciens pur operon promoter, and the resulting fusion was integrated into the yicM gene of AJ1991. Study of the extracellular nucleoside accumulation by the resulting strains showed that the enhancement of phuE expression notably increased insine and guanosine accumulation by AJ1991. We also found that the heterogeneous expression of the E. coli nepl gene in AJ1991 improved the nucleoside productivity of this strain.

We demonstrated that the substrate specificity of the PhuE pump also extends to the AICA ribonucleoside. Overexpression of the PhuE efflux pump in strain AJ1991 ApurE resulted in increased extracellular accumulation of this compound by the strain. The phosphorylated derivative of this compound, AICAR (aminimidazole-4-carboxamide ribonucleotide), acts in cells as an AMP-activated protein kinase agonist and could be an interesting target for biotechnology.

The present data demonstrate that identification and enhancement of the expression of genes whose products facilitate transport of a desired metabolite out of cells could favor the improvement of the respective producing strains for industrial application.

Keywords: purine nucleoside export, AICA ribonucleoside export, marker-free genetic modifications, nepl, phuE, Bacillus amyloliquefaciens

Exploiting S. cerevisiae- and C. elegans-based eukaryotic screening bioassays to diagnose potentially adverse effects of xenobiotics in environmental samples

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The risk associated with the rapidly increasing volume of pesticides being applied in agriculture as well as for industrial and domestic uses is a major concern in ecotoxicology. Synthetic dyes are also, in general, persistent organic pollutants and most of them are xenobiotics that give rise to concerns over ecosystems and public health.

Short-term bioassays, using simple eukaryotic models, are required to make a rapid assessment and warn of potential toxic effects of the bioavailable fraction of the xenobiotics in whole environmental samples. In addition they can be used for preliminary screening of the toxicity of chemicals, e.g. to support high-throughput chemical testing programmes that have been recently launched both in USA and Europe, eventually followed by more complex and expensive testing. Two promising eukaryotic models that also offer the advantage of keeping in line with regulatory limitations to the use of animals in toxicity testing, are the yeast Saccharomyces cerevisiae [1, 2] and the free-living soil nematode Caenorhabditis elegans. The first one is a lower eukaryote easy to manipulate and with a vast amount of genetics knowledge and resources are available. On the other hand, C. elegans has recognized relevance as a test organism for soil and aquatic ecotoxicological studies [3] and it is likely to complement toxicity data obtained with the unicellular yeast model with focus on effects on reproduction and development, neurotoxicity or xenobiotabolism.

To contribute to the development of cost-effective and non-animal alternative screening methods for the toxicological assessment of xenobiotes, we compared two small-scale bioassays to assess the potential toxicity of pesticides and of synthetic dyes. One is based on the inhibitory effects of sub-lethal levels of each xenobiotic on the yeast growth curve using microtiter plate susceptibility assays [1, 2]. The other on their effects on the reproduction of age-synchronized C. elegans worms [3]. Data will be presented on the relative toxicities determined for pesticides from different chemical families and for diverse azo- and antraquinonic dyes, based on the comparison of the lowest-observed-effect-concentration (LOEC) and/or the 50%-inhibitory concentration (IC50) values estimated with both testing systems. Their correlation with ecotoxicity indexes reported in the literature for freshwater organisms will be discussed.

With the aim to identify molecular biomarkers that may be useful for environmental biomonitoring of herbicide toxicity using the yeast model, whole-genome DNA microarrays are being used to examine the global expression profiles occurring in yeast cells in response to sub-lethal levels (e.g. close to the LOEC) of different herbicides. After cells exposure to the IC20 of the chloroacetanilide herbicide alachlor, 97 genes/ORFs showed statistically significant higher levels of transcripts than the control cells not exposed to the herbicide (fold change, FC > 1.5), while 34 were down-regulated (FC < -1.5). A lower number of genes/ORFs (32 and 8, respectively) were differentially expressed in cells exposed to the LOEC that mostly overlapped with the set of IC20-induced or -repressed genes. A total of 15 genes whose expression was modified between 2.5- and 46-fold and that are within biological function categories that are significantly enriched in our data-sets when compared to the entire yeast genome (p value < 0.01), were selected as possible candidates as biomarkers of alachlor exposure. Determination of the dose-response and exposure time-dependency of the expression of these genes is under way, based on real-time quantitative PCR, in order to get a clearer picture of their possible relevance to assess the herbicide toxicity in environmental samples.

Acknowledgments: to FEDER and FCT, Portugal (contracts PITA/AMB/84230/2006 and PITB/BIO/72108/2006)

References:


Keywords: Toxicity bioassays; eukaryotic models; environmental biomonitoring; transcriptomics
Fermentation of fructo- and xylo-oligosaccharides by probiotic and butyrate-producing bacteria

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Fructo-oligosaccharides (FOS) and xylo-oligosaccharides (XOS) are respectively established and candidate prebiotics, widely studied on their ability to stimulate the growth and activity of health-promoting bacteria present in the human gut. The original definition of prebiotic - “a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health” - was synonymous of a selective increase in the growth and/or activity of preferentially hildobacteria and/or lactobacilli. Presently, this definition was broadened - “a prebiotic is a non-viable food component that confers a health benefit on the host associated with modulation of the microbiota” - and it is assumed that there are other key species which are relevant in the saccharolytic fermentation within the colon. Butyrate-producing bacteria are included in this group, since it is recognised that butyrate plays an important role in the maintenance of colonic health.

In this study, the kinetics and metabolism of pure cultures and co-cultures of Bifidobacterium adolescentis DSM 20083 and Clostridium butyricum DSM 10702 on FOS or XOS supplemented media were assessed. Bacterial growth, oligosaccharide (OS) consumption, and short chain fatty acids (SCFA), formate and lactate production were monitored.

The pure cultures and co-culture of B. adolescentis and C. butyricum revealed distinct growth kinetics, different SCFA and lactate production profiles and OS consumption patterns, on each carbon source investigated. Both strains were able to efficiently ferment and grow on FOS, in co-culture and in monoculture. In contrast, the monoculture of C. butyricum revealed a poor assimilation of XOS. In co-culture fermentations, B. adolescentis and C. butyricum were able to positively interact, producing significant amounts of butyrate both on FOS or XOS. This might indicate the existence of cross-feeding between B. adolescentis and C. butyricum, which is especially relevant in the case of XOS supplemented media.

Keywords: Fructo-oligosaccharides, Xylo-oligosaccharides, butyrate, prebiotic

References

Formate dehydrogenases and hydrogen metabolism in Desulfovibrio vulgaris Hildenborough

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Desulfovibrio vulgaris Hildenborough (DvH) is a model organism for the study of Sulfate-Reducing Bacteria (SRB). The sulfate respiratory chain is still poorly understood and there seems to be several pathways involved in the energy conservation, such as hydrogen or CO cycling. Hydrogen is an important energy source for SRB in natural habitats, and previous studies showed that growth with H2 leads to the up-regulation of formate dehydrogenases (Fdh) and pyruvate-formate lyase in DvH [1], suggesting that formate cycling provides an alternative pathway for energy generation.

The DvH genome codes for three different Fdhs. In this work we show that the formate dehydrogenase activity of cells grown in H2/sulfate increases several fold over lactate-grown cells. We isolated the three DvH Fdhs, including FdhaABC3, FdhaAB, a soluble heterodimeric protein, and the soluble subunits of the membrane-bound Fdh, CfdABCD. The three Fdhs show significant differences in activity profile. The growth conditions, including metal composition of the culture medium, influences the expression profile of the three Fdhs.

Keywords: formate dehydrogenases, formate cycling, hydrogen

Functional Interaction Between GacA and Fur in Virulence Regulation of *Pseudomonas syringae* pv. *tabaci* 11528

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In *Pseudomonas syringae* pv. *tabaci* 11528, Fur (ferric uptake regulator) and GacA (global activator of cyanide and antibiotic production) are crucial global regulators, which are known to operate in a variety of other cellular processes. To identify the functional interaction between Fur and GacA in the virulence regulation of *P. syringae* pv. *tabaci* 11528, we conducted phenotype assays with a *fur* deletion mutant (BL33) and a *gacA* deletion mutant (BL473) of this plant pathogen. The results revealed that Fur and GacA coordinately regulate important virulence traits of *P. syringae* pv. *tabaci* 11528. In addition, using a quantitative real-time RT-PCR (RT-qPCR), we determined roles on the expression of several virulence-associated genes by Fur and GacA. Our results indicated that expression of the virulence genes is coordinately regulated by Fur and GacA. Thus, the regulation mechanisms of these global regulators were investigated by an electrophoretic mobility shift assay (EMSA). Consistent with the results of RT-qPCR, we showed that Fur and GacA directly regulate the virulence-related genes at the transcriptional level, respectively. These findings provide genetic evidence of the functional interaction between Fur and GacA in *P. syringae* pv. *tabaci* 11528.

**Keywords** virulence regulation, GacA, Fur, *Pseudomonas syringae*

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Genetic manipulation of the carotenoids biosynthetic pathway by overexpression of the phytoene synthase enzyme in the microalga *Chlamydomonas reinhardtii*.

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The enzyme phytoene synthase catalyses the first step in the carotenoids synthetic pathway in which the 20-carbon GGPP is converted into the first uncoloured carotenoid, phytoene. This is the first specific reaction to the carotenoids biosynthesis and has been regarded as a regulatory key and limiting step in various higher plants species. In microalgae, such as *Chlamydomonas*, the limiting-step role of phytoene synthase was still unclear, but we have shown that its overexpression by introducing additional copies of endogenous or exogenous *psy* genes in *Chlamydomonas* genome can lead to an increase in the carotenoid content.

In the present work we have isolated the genes encoding the *psy* of two microalgae, the model chlorophyte *Chlamydomonas reinhardtii* and the halotolerant carotene-hyperproductive *Dunaliella salina*. The functionality of these genes was checked by complementation experiments in bacteria. The genes were then subcloned into the algal expression vector *PSH105* carrying the appropriate transipt peptide and the microalga *C. reinhardtii* was transformed with the obtained constructions. Several colonies containing the additional *CrPsy* or *DsPsy* genes were isolated and their content in carotenoids were analysed by HPLC. In all cases we found an important increase in the intracellular concentration of several carotenoids, mainly lutein, violoxanthin and β-carotene, which intracellular level increased between 40 to 60% up from its level in control untransformed microalgal cells. The stability of the transgenes and the response of the transgenic lines obtained against several stressing conditions are now under study.

**Keywords** carotenoids; microalgae; *psy*, phytoene synthase *Chlamydomonas reinhardtii*.

**Acknowledgements**: We thank the Spanish Ministry of Education for financial support (AGL2007-6310-C02-01)
Genomic variability of *Thiomonas* sp. isolated from arsenic-rich environments

Florence Arsène-Ploetze1, Sandrine Koechler1, Marie Marchal1, Jean-Yves Copper1, Violaine Bonnefoy3, Fabienne Battaglia-Brunet2, Odile Brunel2, Christopher G. Bryan1†, Jessica Cleiss1, Audrey Heinrich-Krishna Kant Sharma and Ramesh Chander Kuhad

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Arsenic is widely distributed in the environment, released from both natural and anthropogenic sources. Its inorganic forms are highly toxic and impair the physiology of most higher organisms. To get insight into molecular mechanisms of arsenic metabolism in *Thiomonas* strains: differences revealed diverse adaptation processes. BMC Microbiol 9: 127.


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Key Word: Arsenic; Transformation; *Ganoderma* sp.

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**Genetic Transformation of Lignin degrading fungi facilitated by Agrobacterium tumefaciens**

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*Agrobacterium tumefaciens*, a soil phytopathogenic bacterium, generally transforms plants by delivering a portion of the resident Ti- plasmid, the T-DNA (transferred DNA), to plant. *A. tumefaciens* has played a major role in the development of plant genetic engineering and basic research in molecular biology, accounting for 80% of the transgenic plants produced so far. Initially, it was believed that only dicotyledons, gymnosperms and a few monocotyledonous species could be transformed by this bacterium; but recent achievements totally changed this view by showing that many 'recalcitrant' species not included in its natural host range can also be transformed.

Here we describe an efficient and convenient *Agrobacterium* mediated gene transformation system for successful delivery of T-DNA, carrying the genes coding for β-glucuronidase (*uidA*), green fluorescent protein (*gfp*), and hygromycin phosphotransferase (*hpt*) to the nuclear genome of lignin degrading white-rot fungi such as *Phanerochaete chrysosporium*, *Ganoderma* sp. RCKK-02, *Pycnoporus cinnabarinus*, *Crinipellis* sp, *Pleurotus sajor-caju* and BHR-UDSC (Scheme 1). The fungal transformants were confirmed by PCR and southern hybridization. The expression vector pCAMBIA 1304-RCKK was constructed by the addition of GPD promoter from plasmid p416 to the binary vector backbone pCAMBIA1304, which controls *uidA* and *gfp* gene. Transmission Electron Microscopy (TEM) analysis revealed the attachment of bacterial cells to the fungal hyphae. Transformation frequency upto 75% was obtained depending on the fungal species used in the transformation experiments. The transfer efficiency was maximum at 20 °C whereas no transfer was observed at temperature above 30 °C.

**Key Word**: Agrobacterium; Transformation; *Ganoderma* sp.

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**Genome variability of *Thiomonas* sp. isolated from arsenic-rich environments**

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**Key Word**: Agrobacterium; Transformation; *Ganoderma* sp.
Glucansucrase-derived prebiotic oligosaccharides enhance enzyme activity in *Bifidobacterium*

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The objective of this study was to determine the influence of alternansucrase-derived prebiotic oligosaccharides on enzyme activity in the beneficial colonic microbe *Bifidobacterium adolescentis*. Other carbohydrates were included for comparative purposes. Alternansucrase is a bacterial enzyme used to make prebiotic oligosaccharides and sweeteners. Fundamental knowledge of enzyme activity in *Bifidobacterium* may aid in the design of more effective prebiotics and may also help identify indicators of general metabolic activity when assessing their influence within the colon. Activities for α- and β-galactosidase and α- and β-glucosidase were determined from cell extracts of *B. adolescentis* grown on 18 test carbohydrates including alternansucrase-derived oligosaccharides. α and β-galactosidase activities were enhanced on a variety of α- or β-linked carbohydrates regardless if a galactoside or glucoside. α-Glucosidase, however, was enhanced only on α-carbohydrates. β-Glucosidase activity was not enhanced on any carbohydrate tested except for melibiose. Alternansucrase-derived oligosaccharides significantly enhanced α-galactosidase and α-glucosidase activities compared to most of the carbohydrates tested. Most of the alternansucrase-derived oligosaccharides showed significant increases in enzyme activity versus their corresponding acceptor carbohydrates. α- and β-galactosidase may serve as biomarkers for microbial metabolic activity within the colon for potential prebiotics composed of α- or β-linked oligosaccharides whereas α-glucosidase activity may be restricted to assessing the influence of only α-linked carbohydrates. β-Glucosidase would probably not serve as a biomarker for microbial metabolic activity and does not seem to be significantly involved in carbohydrate degradation. In conclusion, the alternansucrase oligosaccharide synthesis process provided a value-added component to carbohydrates by increasing metabolic activity over certain acceptor carbohydrates.

**Keywords** *Bifidobacterium; prebiotic*

Induction of DNA double strand breaks and branched DNA replication structures does not predict the antimicrobial effect of thymidylate deprivation

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Thymidylate deprivation brings about “thymineless death” (TLD) in prokaryotes and eukaryotes. TLD is the phenomenon in which exponentially growing cell starved for thymine loses viability. TLD has been researched over five decades, but the precise mechanism remains elusive. Nevertheless, the enzyme thymidylate synthetase (TS), which catalyzes the *de novo* synthesis of TMP, has served for many years as a basis for chemotherapeutic strategies. To get insight into the mechanism of TLD we use *Escherichia coli thyA* deficient strain with is defective for the enzyme TS, requiring thymine or thymidine for growth. In this system TLD is promoted just by removing thymidylate from the growth medium.

Numerous studies have identified a variety of cellular responses associated to thymidylate deprivation; most of them related with replicating cells. In this work we show that replication forks are required for TLD, but no replication process. Our results indicate that even though, DNA chromosomal replication process is not a requirement for TLD, the amount of forked DNA structures generated under thymidylate deprivation correlates with the significance of the lethality. Since stalled replication forks are generally viewed as promoting double strand breaks (DSBs) that could be the cause of death, we have investigated whether the lethal effect of thymidylate deprivation could be caused by the increase of the amount of DSBs generated by the presence of stalled replication forks (among others mechanisms).

Furthermore, branched DNA created by stalled replication fork could generate entangled DNA that has been associated to TLD and defined as non migrating DNA (mmDNA).

By using pulse field electrophoresis gel (PFEG) we show that DSB and mmDNA are induced by thymine starvation but, i) the amount of DSBs in recB strains doesn’t correlate with TLD, as the DSBs persisted after rifampicin addition- condition where TLD was suppressed ii) the presence of non migrating DNA that remain in the well after being digested with XbaI does not agree with TLD, as mmDNA was not observed after hydroxyurea addition- condition where TLD is not affected. We propose the forked DNA as the key element for TLD. In this sense, DSBs and mmDNA would be required but they wouldn’t be sufficient for the cell to undergo TLD.

**Keywords** thymidylate, thymineless death; fork, DNA replication.
Influence of Hap2p and Hap3p on KIHI4 promoter binding activity

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Analysing the KIHI4 promoter by gel retardation assays, we characterised a specific retarded band, Fc1, whose intensity is carbon-source dependent in a hap2p mutant background. This band becomes evident in protein extracts from a KIHI2P2 knocked-out strain. Although the kihap3 mutation is not able to enhance the band formation by itself, the combination of kihap2 and kihap3 mutations has a synergistic effect on the binding activity. In KIHI4 promoter we found a CCAAT box, a putative binding site for the Hap complex but mutations in this site showed that the Fc1 band intensity is not dependent of this sequence. KIHI4 mRNA levels in the double kihap2/3 mutant do not correlate with the increase in Fc1 binding activity, indicating that the role of Fc1 on the promoter is not KIHI4 transcriptional activation.

This is the first evidence that the KIHIp complex, homolog to Saccharomyces cerevisiae Hap2p/3p/4p/5p activator complex, acts by blocking a carbon-source-dependent binding activity. These data allow to a better understanding of the differences in expression of homologous genes regulated by this complex, including genes related to respiro-fermentative metabolism previously reported.

Keywords: yeast, hap2/3/4 complex, transcription, Kluyveromyces

Influence of inoculum and residual glucose concentration on Streptococcus pneumoniae serotype 6B cultivation

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Introduction: Streptococcus pneumoniae is a major pathogen commonly responsible for pneumonia, bacteremia, meningitis and otitis media, especially among young children and elderly. The most prominent pneumococcal virulence factor is the capsular polysaccharide (PS), which coats the surface of the bacterium and act as an anti-phagocytic factor “in vivo”. S. pneumoniae express at least 91 distinct capsules which are chemically and serologically distinct. The capsule is currently used as antigen in pneumococcal vaccines, either as free polysaccharide or as PS conjugated to proteins. The conjugated vaccines enhance PS immunogenic character, inducing T-dependent immune response and immunological memory. Pneumococcal serotype 6B is the second most prevalent in Brazil. The optimization of cultivation conditions is part of a pneumococcal vaccine development project of Instituto Butantan In this project the three most prevalent pneumococcal serotype will be conjugate to a protein Pneumococcal Surface Protein A (PspA).

Objectives: In the present study we investigated the influence of both: the growth phase of the inoculum and the residual glucose concentration at the instant of the pulse or the start of feeding on capsular polysaccharide (CPS) production in batch and fed-batch cultivation.

Materials and Methods: Streptococcus pneumoniae serotype 6B strain ST 433/03 was used. Bench scale experiments were carried out using a variant of the Hoeprich medium, containing glucose, acid-hydrolyzed casein, dialyzed yeast extract, L-glutamine and asparagine as nitrogen sources, choline as a growth factor and salts. The inocula were cultivated in this medium and incubated in a candle jar at 37°C for 13h. The volume inoculated into the reactor varied in order to set the initial OD around 0.1 at 600nm. The experiments were conducted in 5L-bioreactors (New Brunswick Scientific Inc.), at 37°C and 200 rpm; with controlled pH at 7.0 and polypropylene (PPG) - an antifoam agent - was added as required. The bioreactor was monitored by the LabView 7.1 program (National Instruments). Nine experiments were carried out in batch with a pulse of 100mL of 50% w/v glucose and 45mL of 46.2% w/v ammonium acetate. And six experiments were carried out in fed-batch.

Results and Discussion: According to the inoculum growth curve, a culture with an OD=1.6 was in the mid-log phase and a culture with an OD=2.6 was at the end of the log phase. In batch cultivation, it was observed that the lower was the OD of the starter culture, the higher was the PS concentration obtained. The residual glucose concentration at the moment of the pulse also influenced the PS production: PS production was higher when the pulse was given at higher residual glucose concentration. Hence, in batch culture the highest PS production (387 mg/L) was obtained using an OD=1.6 of the starter culture and giving the pulse when the residual glucose was ~15 g/L, while using a similar inoculum (OD=1.65), the PS concentration reached 248 mg/L after giving the pulse when the residual glucose was 4.5 g/L. The lowest PS production was obtained when a culture with an OD=2.6 was inoculated into the reactor and the pulse was given when the residual glucose was 3.4 g/L (PS=194 mg/L).

A similar relationship between OD of the inoculum and PS production was obtained in fed-batch cultures: the highest PS concentration (393 mg/L) was obtained when the reactor was inoculated using a culture with OD=1.6 and the lowest PS production (198 mg/L) was obtained by using a culture with OD=2.4. The effect of the residual concentration at the instant of the start of feeding on PS production was probably influenced by the presence of other components in the concentrated feeding medium, which could better fit the nutritional requirements of the microorganism.

Inoculum preparation is the part of the fermentation development process that secures the microbial phenotype for experimental and/or production purposes by providing a viable biomass capable of high productivity. It is certain that the inoculum preparation stage of most fermentation will affect the expression of the desired phenotype, whether qualitatively, quantitatively, or temporally. Hence, inoculum development is faced with a fundamental problem.

Conclusions: The physiological state of the inoculum showed an important correlation to the PS production in batch and fed-batch cultivation of S. pneumoniae serotype 6B: mid-log phase inocula yielded high PS production. In batch production, it was also observed a synergic effect of the inoculum OD and the residual glucose concentration in the moment of the pulse on PS production. These phenomena are consequence of the growth profile and the action of lytic enzymes after the log phase should be involved. Financial Support: FAPESP.
Influence of the Mth genes on synthesis of alcohol oxidase and catalase in methylotrophic yeasts Pichia methanolica.

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Some methylotrophic yeast mutants and their revertants, incapable of assimilating methanol and with much lower alcohol oxidase activity (AO) have been under investigation. Using analysis of molecular isozyme forms we researched the product activity of the genes, which are possible homologue of Saccharomyces cerevisiae peroxisome catalase - CTA1, and AUG2, coding one of alcohol oxidase subunits. The conditions favorable for the gene of AUG2 expression (3% methanol in growth medium) lead to increased synthesis of peroxysome catalase. And on the contrary, the unfavorable conditions (without carbon source or with 1% glycerol) caused the decreased level of the peroxysome catalase. The mth1/mutant mainly had dominant formation of AO isofrom with electrophoretic mobility which is typical to isogenic form 9, the product of the gene AUG2, and decreased level of peroxysome catalase. Four spontaneous revertants of the mutant mth1 (Rmth1) restored their growth on methanol which was accompanied both with increasing activity of AO isogenic form 9, the product of the gene AUG2, and peroxysome catalase. The obtained results confirm the existence of general regulatory elements under the gene AUG2 and CTA1 function at some stages of regulation in methylotrophic yeasts P. methanolica.

This work was supported by the Russian Foundation for Basic Research, grants № 08-04-01691-a and № 09-04-90360-S-Осет-a.

Isolation, characterization and regulation of carotenoid biosynthetic pathway genes in microalga

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Carotenoids are produced by all photosynthetic species and several not photosynthetic organisms playing roles in light-harvesting, photoprotection, structural maintenance of pigment-protein complexes, and membrane structure and fluidity. The specific carotenoid biosynthetic pathway starts with the condensation of two geranylgeranyldiphosphate molecules to produce phytoene, the first carotenoid. Four desaturation reactions convert phytoene into lycopene, which is modified to β-carotene by the action of lycopene β-cyclase (LCYb) or into α-carotene by the action of LCYb and lycopene ε-cyclase (LCYe). The cyclization of lycopene to α- and β-carotene is a key branch point in the pathway of carotenoid biosynthesis and has proved to be a control step in the biosynthetic pathway. α-carotene is modified to lutein by two hydroxylases and a limited number of organisms including some green algae as Haematococcus pluvialis and Chlorella zofingiensis can synthesize astaxanthin from β-carotene by the action of a ketolase/oxygenase and a hydroxilase. Astaxanthin and lutein are high value carotenoids which are not only used as food dyes and as feed additives in aquaculture and poultry farming, but also are considered as effective agents for the prevention of a variety of age-related, degenerative and chronic diseases, as cataracts, macular degeneration, cancer and atherosclerosis.

Our group has carried out the isolation, characterization and the study of the regulation in response to irradiance and nitrogen limitation of the lcyB gene of C. zofingiensiis. By using RT-PCR and RACE-PCR a 2131 bp cDNA with a 1641 bp open reading frame was isolated in this microalgae. Alignment of this cDNA with the corresponding genomic sequence revealed the presence of 7 exons and 6 introns in the lcyB gene, which encodes a hypothetical protein of 546 amino acids with an estimated molecular weight of 60 kDa. Homology studies have shown that the deduced amino acid sequence has a high homology with sequences of other microalgae and higher plants and lower with cyanobacterial, and very low with bacterial sequences. Hypothetical FAD binding domain present in other plant and microalgal lycopene b-cyclases has also been found in the C. zofingiensis LCYb sequence. Southern analysis with genomic DNA has indicated that the C. zofingiensis lcyB gene is present in a single copy. The functional analysis by plasmid transformation in E. coli has shown that the protein allows the double cyclation of lycopene to produce β-carotene and the formation of a new β-cycle in the monocycled δ-carotene to yield α-carotene. By quantitative Real Time PCR no differences in lcyB transcript levels have been observed in C. zofingiensis cells growing photosynthetically at both high and low irradiance. However, nitrogen depletion increased significantly transcript levels at all irradiances.

We are also interested and working on the isolation and characterization of other genes of the carotenoid biosynthetic pathway. We also have partially isolated the phytoene synthase gene of C. zofingiensis. Phytoene synthase is the first enzyme of the carotenoid pathway and therefore could play an important control role in the carbon flux toward carotenoid synthesis. Currently, we are trying to isolate the LCYe gene of C. zofingiensis by the design of degenerated primers based in the conserved motifs of different known LCYe from algae and plants. Moreover, we are studying the regulation of the carotenoid pathway in H. pluvialis by analysing the expression patterns of the main carotenogenic genes and the carotenoids accumulation profiles under different stress conditions such as, high irradiance, nutrients deficiency and salts stress.

This work has been supported by Ministerio de Educación y Ciencia, Spain (grant AGL 2007-65308-C02-02), and Junta de Andaluca, group BIO-299.

Keywords carotenoids; astaxanthin; lutein; Chlorella zofingiensis; Haematococcus pluvialis; carotenoid biosynthesis genes
Mechanisms of SigH activation in minor cell population: a stochastic process or gene activation by short junction duplication

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Staphylococcus aureus naturally inhabits on human nasal cavity, but it is also an opportunistic pathogen. It is equipped with a variety of stress tolerance/adaptation mechanisms, which are modulated by sigma factors or other transcription factors. The main focus in this presentation is on an ordinary silent sigma factor gene, sigH, whose physiological function is still unclear. Interestingly, it can be activated only in minor cell population. The SigH active cells can be selected by using a tetracycline resistance reporter system. The frequency fluctuates from $10^{-5}$ to undetectable level ($<10^{-9}$). This activation depends on the duplication of the sigH locus generating a new chimeric gene. The SigH activity diminishes at frequencies of $10^{-2}$ ~ $10^{-3}$, in concomitant with the disappearance of the amplified unit. Another line of experiments using GFP reporter shows that SigH is stochastically activated under certain culture condition with higher frequencies ($10^{-2}$) probably through the post-transcriptional regulation, indicating that there are multiple activation mechanisms. The agr quorum sensing system had a positive role in the stochastic SigH activation, but was not essential. The physiological significance of such SigH activation modes will be discussed.

Keywords: Staphylococcus aureus, sigma, stochastic expression, Short Junction duplication

Molecular characterization of light sensitive mutants of the microalga Chlamydomonas reinhardtii

M. Vila, I. Conso and R. León

Insertional mutagenesis has demonstrated to be a powerful tool to study the relationship existing between a gene sequence and its function in the microalga Chlamydomonas reinhardtii. The technique is based in nuclear transformation of the microalga with an exogenous DNA marker, which is integrated randomly into the genome. After screening for a particular phenotype and analysis of the disrupted genes it is possible to establish a functional relation between the affected gene and the phenotype selected. This approach has allowed identification of many mutants affected in many different aspects (Galván et al., 2007). And has become a popular method for forward genetics studies because identification of the affected genomic region is generally easier that location of genomic lesions caused by traditional mutagenesis procedures based on chemical or physical agents.

In the present work we have selected Chlamydomonas reinhardtii mutants sensitive to high light intensities using AphVIII gene that confers resistance to the antibiotic paramomycin as marker gene. Chlamydomonas cells were transformed by agitation with glass-beads method with the cassette pBhcS2-AphVIII-erRho2. 1300 Insertional mutants were isolated in the presence of paramomycin and submitted to a basic screening to isolate light-sensitive or pigment deficient mutants. We found five high-light sensitive mutants. The genomic DNA of the selected mutants was isolated and submitted to a special PCR named enzyme site directed amplification PCR (RESDA-PCR) (González-Ballester et al., 2005). The amplified bands were sequenced and compared with the genome sequence (version 4.0) of Chlamydomonas (http://genome.jgi-psf.org/Chlre4/Chlre4.home.html). This technique allowed us to identify the genomic region adjacent to the marker DNA used for transformation in four of the obtained mutants. All the identified sequences showed similarity with Chlamydomonas genome sequence. Detailed analysis of the affected genes at its relation with the observed phenotype is being carried out.

References


Keywords: microalgae, light-sensitive mutants, insertional mutagenesis, Chlamydomonas reinhardtii

Acknowledgements: We thank the Spanish Ministry of Education for financial support (AGL2007-65303-C02-01)
Multiple promoters of stress-responsive genes of *Corynebacterium glutamicum*

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Promoters of stress-responsive genes of gram-positive amino-acid-producing bacterium *Corynebacterium glutamicum* have been characterized. Potential promoter regions of selected genes were cloned into the promoter-probe vector pET2 upstream of the promoterless cat reporter gene coding for chloramphenicol acetyltransferase (CAT). The promoter activity was assayed as activity of reporter enzyme CAT during standard cell growth at 30 °C and after heat shock (40 °C, 1 h) and cell surface stress (growth with 0.01 % SDS or 4 mM EDTA for 90 min) in *C. glutamicum* wild-type strain as well as in *C. glutamicum* ΔsigH, ΔsigE and ΔsigM mutant strains with deleted genes coding for the stress-responsive sigma factors of RNA polymerase. Promoter activities of promoter regions upstream of *C. glutamicum* sigH (coding for alternative sigma factor), arnC (coding for small untranslated regulatory RNA), clpB, clpC, dnaJ2 genes and dnaK-grpE-dnaJ2-hspR operon (coding for chaperon proteins and a regulator involved in stress response) significantly increased after heat shock in *C. glutamicum* wild-type strain, while in *C. glutamicum* ΔsigH, promoter activities of these regions were significantly lower. Promoter activity of the region upstream of the sigE gene, coding for an alternative sigma factor, strongly increased after cell surface stress.

Using non-radioactive primer extension (PEX) method, multiple (2-4) transcriptional start points (TSPs) were determined within the upstream regions of all tested *C. glutamicum* stress-responsive genes and the respective -10 and -35 hexamers of the putative promoters were proposed. The major TSP of sigE gene was mapped at the base A, located within the translation initiation ATG codon, while the other TSP, detected only after SDS treatment, was localized in the position -14 upstream of translation initiation codon. Since no effect of deletions of genes coding for stress-responsive sigma factors (sigD, sigE, sigH or sigM) on promoter activity was observed, both promoters P-sigE1 and P-sigE2 are most probably recognized by the primary sigma factor SigA.

Sequences highly similar to consensus sequences of promoters recognized by stress-responsive sigma factors (GGAAN$_{-20}$GTT) were detected in -35 and -10 regions pertinent to some of TSPs of the heat-shock genes (arnC, sigH, clpB, clpC, dnaJ2 and dnaK-grpE-dnaJ2-hspR operon), in addition to the -10 hexamers highly similar to the consensus sequence of vegetative *C. glutamicum* promoters recognized most probably by the primary sigma factor SigA. Transcription starting at two TSPs of dnaJ2 and at one TSP of sigH was found to be strongly reduced in *C. glutamicum* ΔsigH after heat shock, when compared with the wild-type strain. These results are in agreement with the results of reporter CAT assays and suggest that SigH is directly involved in transcription of *C. glutamicum* sigH and dnaJ2 genes after heat shock.

Multiple promoters of stress-responsive genes in *C. glutamicum*, recognized by different sigma factors, represent important regulatory elements in cell response to environmental stress factors.

**Keywords:** *Corynebacterium glutamicum*, stress response, promoters, sigma factors

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**Plant Cell Contact-dependent Virulence Regulation of hrp Genes in Pseudomonas syringae pv. tabaci 11528**

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*Pseudomonas syringae* is a widespread and representative plant pathogen that can colonize the intercellular spaces of aerial organs. *P. syringae* pv. *tabaci* causes wildfire disease in tobacco plants. As a means to facilitate the isolation of *P. syringae* pv. *tabaci* 11528 from this library, and then subjected to shotgun sequencing. Pathogenicity of *P. syringae* pv. *tabaci* 11528 is dependent on the type III secretion system (T3SS), which is encoded by hrp PAI and required for injection of large repertories of virulence effectors into host cells. It can be induced in minimal media which mimic plant apoplastic fluids, and the induction depends on the sigma factor HrpL. However, recent studies provided evidence that specific host factors induce the expression of the *Ralstonia solanacearum* hrp PAI. This specific plant cell induction of hrp PAI is controlled by PrhA (plant regulatory of hrp), a protein that shows homology to outer-membrane siderophore receptors. In this study, *prhA* homologues of *P. syringae* pv. *tabaci* 11528 and *P. syringae* pv. *tomato* DC3000 were amplified by PCR, and *ΔprhA* mutants were constructed by allelic exchange. The *prhA* gene was shown to encode a protein of 722 amino acids, while *PrhA* revealed significant similarities to numerous TolI-dependent siderophore receptor proteins. In several physiological analyses (growth rate, swelling motility, CAS universal siderophore detection assay), the *PrhA* mutants showed differences from the wild type strains. We tested whether *prhA* does regulate the transcription of *hrpA*, by comparing the level of β-galactosidase activity after the cell culture in a *hrp*-inducing minimal medium. Although there was no difference in the level, *aprHa* mutants reduced virulence in host plant. Therefore it was postulated that the PrhA of *P. syringae* is a putative pathogen-plant cell contact sensor, and a *hrpA* reporter gene fusion was employed to prove it. Co-culture of *P. syringae* and *Arabidopsis thaliana* or *Nicotiana tabacum* (tobacco) cell suspensions resulted in much higher induction levels of *hrpA* gene transcription than those obtained in *hrp*-inducing minimal medium. This work provides evidence that the recently characterized plant-responsive regulatory cascade induces *hrp* gene expression in *P. syringae* in the presence of plant cells.

**Keywords:** *Pseudomonas syringae*, hrp pathogenicity island; prhA; virulence regulation
Proteomic Analysis of the Responses to Phenol Concentration Variations in Moderately Halophilic Bacteria *Halomonas* sp. MU12

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Phenol is one of the main pollutants found in the industrial wastewaters because of its widespread usage in the oil refining, pharmaceutical and resin manufacturing plants. The presence of phenol in the environment poses a significant risk to aquatic biota even at low concentrations. Removal of phenol from wastewaters is therefore an important issue. Although, physicochemical methods are used generally to remove phenol from industrial effluents, these treatments are usually expensive and complex. Comparing to chemical methods, biotreatment of phenol-containing wastewater is a low cost process and it has the possibility of complete mineralization of phenol.

Halophiles are an important group of microorganisms that can adapt to extremely saline environments. Moderately halophilic microorganisms have been in the center of industrial interest in the last decades owing to their growth in wide range of salt concentration.

In this study, responses to phenol concentration variations in moderately halophilic bacterium *Halomonas* sp. MU12 isolated from Çamaltı Salt Area of Türkiye was investigated. Strain MU12 has an ability to grow between 5 % and 25 % NaCl concentrations. Proteome analysis is conducted by means of a two-dimensional polyacrylamide gel electrophoresis (2D PAGE), and matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS) was conducted to obtain the adaptive responses inside the bacterium. The MU12 cells grown to the exponential phase in both 200 mg.L\(^{-1}\) and 1000 mg.L\(^{-1}\) phenol containing medium were harvested and proteins were extracted for proteome analysis. Comparative analysis of the proteome profiles of strain MU12 grown in 200 mg.L\(^{-1}\) and 1000 mg.L\(^{-1}\) phenol allowed us to identify all the proteins involved in the phenol adaptation metabolism.

This project was supported by Marmara University Scientific Research Center with a project no FEN-C-DRP-181205-0286.

**Keyword:** phenol adaptation metabolism, proteomics, moderately halophiles

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Proteomic analysis of antibiotic resistance in *Salmonella* spp. strains from wild rabbits and boars

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*Salmonella* is a Gram-negative, facultative intracellular and food-borne bacterium that causes gastroenteritis and typhoid fever in humans. *Salmonella* infections appear to be one of the most typical examples of enteric diseases transmitted from animals to humans. With more than 2,000 serovars, *Salmonella* also comprinds pathogenic enterobacteria and some of its serotypes, mainly *S. enteritidis* and *S. typhimurium*, presenting as a major cause for food intoxications constituting a serious problem of public health.

Proteomics comes as a very important area on knowing the genes function and their products, as well as understanding the proteins involvement in specific cellular processes. Bidimensional electrophoresis (2-DE) combined with aminoacid sequencing by mass spectrometry has a fundamental role about understanding antibiotic resistance mechanisms in *Salmonella* spp. isolated from faecal samples of wild animals (rabbit and boar) from the North of Portugal.

Subsequently to extraction and protein solubilization of the bacterial strains, samples were submitted to IEF electrophoresis (isoelectric focusing) in 13 cm long IEF strips pH 4-7 and afterwards to a second dimension obtained in SDS-PAGE gels at 12.5%, 2-DE gels were then stained in Coomassie G-250 with posterior image analysis supported by Melanie 5.0 software for suitable protein spots cataloguing. A manual and individualization collection of 14 protein spots was conducted for later trypsin digestion and MALDI-TOF.

After aminoacid sequencing of each spot and comparing the obtained data with bioinformatic databases it was possible to determine the specific peptides present, with a high protein score confidence interval. In the protein spots identified, several were related to different *Salmonella* enterica serovars (Typhi, Typhimurium, Paratyphi A, Paratyphi B and others) normally associated to humans gastroenteritis and salmonellosis. The presence of proteins related to *Salmonella* serovars related to infectious processes in humans with possible transmission from infected animals, brings to question a major public health problem when considering that these animals can be in contact with domesticated animals or even humans.

The complete proteome annotation of this bacterial strain isolated from wild rabbits and boars from the Trás-os-Montes and Alto Douro will allow to increase the knowledge on the bacterial resistance rate in wild animals.

**Table 1. Comparison of the protein identification of selected spots through MALDI-TOF.**

<table>
<thead>
<tr>
<th>Spot</th>
<th>Protein Description</th>
<th>Species</th>
<th>Protein Name</th>
<th>Accession Number</th>
<th>Protein MW</th>
<th>Protein PI</th>
<th>Protein Count</th>
<th>Peptide Count</th>
<th>Protein Score</th>
<th>Protein Score C.I. %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td><em>Salmonella typhimurium</em></td>
<td>gapA</td>
<td>P0AL20</td>
<td>35564,30859</td>
<td>6,33</td>
<td>16</td>
<td>581</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Superoxide dismutase</td>
<td><em>Salmonella typhimurium</em></td>
<td>sodA</td>
<td>P0AL20</td>
<td>23064,90045</td>
<td>6,45</td>
<td>7</td>
<td>323</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Urease</td>
<td><em>Salmonella typhimurium</em></td>
<td>ure</td>
<td>P0AL20</td>
<td>27322,80808</td>
<td>6,08</td>
<td>9</td>
<td>307</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Triosephosphate isomerase</td>
<td><em>Salmonella typhimurium</em></td>
<td>tpiA</td>
<td>P0AL20</td>
<td>26929,88086</td>
<td>5,68</td>
<td>7</td>
<td>250</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Triosephosphate isomerase</td>
<td><em>Salmonella enterica</em></td>
<td>tpiA</td>
<td>P0AL20</td>
<td>26895,85958</td>
<td>5,68</td>
<td>6</td>
<td>237</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Phenylacetate decarboxylase</td>
<td><em>Salmonella enterica</em></td>
<td>pdc</td>
<td>P0AL20</td>
<td>41596,83394</td>
<td>6,09</td>
<td>14</td>
<td>278</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>L-Arginine aminotransferase</td>
<td><em>Salmonella enterica</em></td>
<td>argD</td>
<td>P0AL20</td>
<td>58082,53125</td>
<td>6,58</td>
<td>9</td>
<td>50,1</td>
<td>94</td>
<td></td>
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<tr>
<td>13</td>
<td>Flagellin</td>
<td><em>Salmonella rubidus</em></td>
<td>fliC</td>
<td>P0AL20</td>
<td>31880,90847</td>
<td>4,99</td>
<td>4</td>
<td>83,8</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>
Regulation of ectoine biosynthesis in halotolerant methanotroph

*Methylocibacterium alcaliphilum* 20Z.

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Aerobic halotolerant methanotroph *Methylocibacterium alcaliphilum* 20Z regulates osmotic balance between cytoplasm and surrounding medium by accumulation of osmoprotectant ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidine carboxylic acid) having multiple functions. The pathway of ectoine biosynthesis represents the branch of the biochemical pathway for aspartate family amino acids synthesis and involves three specific enzymes: diaminobutyric acid (DABA) acetyltransferase (EctA), DABA aminotransferase (EctB) and ectoine synthase (EctC). Remarkably, the genes encoding these enzymes in *M. alcaliphilum* 20Z were shown to be organized into the ectABCask operon additionally containing aspartokinase gene ask.

Transcription of the operon in *M. alcaliphilum* 20Z is initiated from two promoters similar to the *Salmonella* paratyphi A rplE. Upstream of the gene cluster a new open reading frame (ectR) encoding MarR-like transcriptional regulator was identified. Investigation of the EctR influence on the activity of the ectABCask operon in wild type and ectR* M. alcaliphilum* 20Z implied that EctR is a negative regulator of the ectABCask operon. The recombinant EctR-His 6 specifically binds as homodimer to the putative -10 motif of the EctAp1 promoter. The EctR binding site contains a pseudo palindromic sequence (T ATTTAGT-GT-ACTATATA) composed of 8-bp half-sites separated by 2bp. Transcription of the ectR is initiated from a single promoter ectRp similar to the *Salmonella* paratyphi B rpsL-dependent promoter of *E. coli*. Location of the EctR binding site between transcriptional and translational start sites of the ectR suggests that EctR regulates its own expression.

Analysis of the DNA fragment containing ectoine biosynthetic genes (EU315063) in methanol-utilizing bacterium *Methylobaeta alcalalis* showed the presence of *orf* with high homology to the *ecr* from *Mm. alcaliphilum* 20Z (73% identity of translated amino acids). Moreover, NCBI Database search revealed ecr-like genes located immediately upstream of the ectoine gene cluster in 17 halophilic bacterial species. Between them, the *orf* of *Oceanospirillum* sp. (ABD80450), *Saccharophagus degradans* (ZP_01114878) and *Oceanobacter* sp. (EAT11341) showed the highest identities of translated amino acid sequences (35.5, 43.2, 45.6, 51.7 and 55.1% respectively). These results clearly evidenced the presence of a new earlier uncharacterized regulatory system controlling ectoine biosynthesis at transcriptional level in diverse halophilic/tolerant bacteria.

Keywords halophilic methanotrophs; ectoine; regulation of biosynthesis; gene transcription
Role of the nemRA operon of Escherichia coli K-12 in reducing ubiquinone as well as glyoxal

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Glyoxal (GO) is a reactive aldehyde compound, generated by oxidation of glucose. Due to its modification of proteins as well as nucleic acids, it is toxic to bacterial cell. There are at least two mechanisms known to detoxify GO, aldo-keto reductases and glutathione-dependent glyoxalase. Previously, we characterized yqhCD genes as a system to remove intracellular GO. To further screen genes related to GO, we isolated novel GO R mutants from yqhD deletion strain. One of the GO R mutations was mapped in the nemR promoter region, which was carried out using TnphoA insertions and their cotransductional linkages. Since NemR and RutR, known as repressors of the nemRA operon, share the binding site in nemR promoter region, the mutation in this site directly affects the DNA binding affinity. Therefore, both repressors are loosely bound to the promoter, and the nemRA operon is constitutively upregulated. We examined enzymatic activities of NemA on GO, methylglyoxal (MG), acrolein, etc using NADPH as a cofactor. In addition, an overproduction of NemA confers GO resistance. NemA is known as a member of FMN containing old yellow enzyme (OYE) family, and its ability of to transfer electron to electrophilic substrates has been reported, although its physiological substrate remained obscure. We tested ubiquinone as its substrate and observed that not only NemA reduces ubiquinone to ubiquinol using NADPH, but also ubiquinone interacts with NemR, dissociating NemR-DNA complex. Therefore, we propose that the nemRA operon is induced by ubiquinone and plays a critical role in generating reduced ubiquinone pool, in addition to its activity in reducing u-oxoaldehydes.

Keywords nemR; nemA; ubiquionone; glyoxyal

ROS production and cell cycle arrest in Saccharomyces cerevisiae during nitrogen-depleted alcoholic fermentation

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In the present work, differences on the physiological state of a wine Saccharomyces cerevisiae strain growing in feed-batch culture under nitrogen deficiency during alcoholic fermentation were evaluated by flow cytometry, in respect to the following parameters: intracellular reactive oxygen species (ROS), plasma membrane integrity and cell cycle. The data obtained indicate that the stress conditions occurring during nitrogen-depleted fermentation resulted in ROS production that increased along fermentation about two-three fold comparatively to the control fermentation. The use of dihydroethidium (DHE) as a probe for detecting the generation of superoxide anion showed a weak contribution of this anion for the overall ROS determined by MitoTracker Red CM-H2XRos, under nitrogen-depleted conditions, indicating that mitochondria are not a major target for nitrogen starvation during fermentation, as also supported by results obtained with rhodamine 123, a specific dye to evaluate mitochondria function. In contrast, the superoxide anion levels largely contribute to the overall ROS observed under control conditions. Nitrogen depletion also induces loss of plasma membrane integrity (evaluated by PI) that followed the same profile of ROS production. Nitrogen re-feeding 72 hours after inoculation into nitrogen-depleted fermentation revert the observed effects on plasma membrane integrity and decreases oxidative stress. In addition, cell cycle analysis revealed that nitrogen depletion induces, after 48 hours fermentation, a persistent arrest of 100% of cells in cell cycle phases G0/G1, comparatively to 60% of cells under control conditions. Nitrogen re-feeding allows cells re-entering cell cycle. These findings indicate that nitrogen depletion during wine fermentations induces oxidative stress associated to plasma membrane damage, cell cycle arrest without a major contribution of mitochondria. Altogether the results provide new insights on the understanding of wine fermentations under different initial nitrogen conditions.

This work was partially supported by FCT through the project POCTI/AGR-ALL/71460/2006

Keywords: Wine yeasts; nitrogen depletion; ROS; Cell cycle; membrane integrity.
Screening of Halobacterium salinarum DNA sequences coding for salt resistance in a yeast model

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In the past few years, the study of the Archaea Domain has revealed similarities to the Eukarya Domain, especially concerning elements involved in transcriptional regulation. Halobacterium species are salt-dependent halophilic organisms, unable to grow in NaCl concentrations lower than 1 M. Therefore, these organisms may be considered a rich source of genes involved in salt tolerance. Taking this into account, the budding yeast Saccharomyces cerevisiae, a well known organism for salt tolerance studies, was used as host to a genomic library of the Archaea Halobacterium salinarum, which was screened on plates containing 1.5 M of NaCl. The library was constructed in a plasmid harboring the strong yeast promoter PGK. Thirty-seven clones of S. cerevisiae able to grow in 1.5 M NaCl were isolated for further studies. Plasmids isolated from these clones were able to increase salt tolerance to new transformed cells and the loss of the plasmid led the host cells to present the original salt tolerance. The plasmid inserts of these selected clones are being currently characterized.

Keywords Archaea, Genomic Library, Salt tolerance

Supported by FAPEMIG CBB APQ-2337-3.12/07

Screening of novel bacteria for biohydrogen production

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The biological production of hydrogen constitutes a promising alternative to fossil fuels based energy sources. Some major advantages of energy from hydrogen are its high energy content, the green nature of its combustion and the possibility of its use in fuel cells. In addition, the possibility of using biowaste materials as major fermentation substrates opens the perspective of adding “environmental value” to the process of biohydrogen production. Lignocellulosic-rich materials, such as agricultural and some industrial residues are inexpensive, renewable and abundant resources, which are particularly suitable for biohydrogen production. However, the process of direct fermentation of such lignocellulosic feedstock is still inefficient, and consequently hinders the possibility of implementing a consolidated bioprocessing strategy, in which enzyme production, substrate hydrolysis, and fermentation are accomplished in a single step by microorganisms that express cellulolytic and hemicellulolytic enzymes [1]. New and improved bacterial strains showing enhanced rates and/or yields of biohydrogen production are needed in order to achieve higher conversion efficiencies and a successful implementation of a “third generation” process of biohydrogen production.

The objective of this work was to characterize hydrogen production by Clostridium butyricum in the presence of different carbon sources and to isolate new biohydrogen producing strains which may more effectively convert carbohydrates into hydrogen.

The effect of different carbon sources was analysed in terms of hydrogen production, substrate concentration, organic acids production and pH. Xylose, which is one of the dominant monosaccharides obtained from saccharification of lignocellulosic feedstock, originated the highest value of hydrogen production among the tested sugars, making up more than 30% of the total gas mixture in the cultures headspace. Using anaerobic cultures techniques, seventeen potential hydrogen-producing isolates were obtained from an anaerobic digestor. These isolates were characterized morphologically and the hydrogen present at the cultures headspace was monitored.

Keywords Biohydrogen, Clostridium butyricum, xylose, lignocellulosic feedstock

Dr. Isabel Paula Marques (UB, LNEG, Lisboa, Portugal) is gratefully acknowledged for supplying the samples from the anaerobic digestor. Luanda Bartolomeu (UB, LNEG, Lisboa, Portugal) is gratefully acknowledged for support on GC analysis.

Structural Instability in Plasmid Vectors for DNA Vaccination

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DNA repeats are considered to be catalysts of molecular evolution by promoting genetic instability and mutational events. One of the numerous concerns associated with direct or inverted repeats consists on the occurrence of associated homologous recombination events within plasmids used for transgene expression or biopharmaceutical applications, namely gene therapy and DNA vaccination (recently reviewed by Oliveira et al., 2009).

In the recent years some authors have found practical evidences for structural instability phenomena occurring spontaneously in plasmids used for DNA vaccination (reviewed by Oliveira et al., 2009). These include direct-repeat mediated deletion-formation events in the vector pCIneo (Ribeiro et al., 2008) or IS3-mediated instability in a vaccine against HIV (Prather et al., 2006).

In this work we present data on the influence of different growth parameters (medium composition, temperature shifts, aeration, absence/presence of selective pressure) and stress intensity (antibiotic concentration) on pCIneo deletion-formation. Furthermore, we show that this commercial vector contains additional instability regions, including a structure-dependent hotspot for IS2 insertion (Oliveira et al., submitted). Predictive models for recombination frequency were also developed that take into account repeat and spacer length (Oliveira et al., 2008).

A computational search for direct and inverted repeated regions with high recombination potential performed in a large sample of commercial vectors, led us to conclude that these hotspots are widespread, even in plasmids currently used for DNA vaccine development. As a result of this in silico analysis, we were able to detect a spontaneous recombination between two 21-bp direct repeats present in the human cytomegalovirus immediate early enhancer/promoter (huCMV IE) of pCIneo. This finding is of paramount importance, as the huCMV IE is one of the most frequently used regulatory elements.

Altogether, these findings are crucial in understanding not only how plDNA instability can be shaped by stressful environment but also the real extension of potentially threatening hotspots.

Keywords: plasmid, DNA vaccine, homologous recombination, transgene expression, repeats

References:

Studies of internal structure of multicellular microbial community by two-photon confocal microscopy

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Principles guiding the development and long-term survival of yeast populations significantly differ from those of shaken liquid cultures. When growing on solid surfaces, yeast form organised multicellular structures, colonies, of unique properties. Cells localised in central colony areas undergo metabolic changes different from those that occur in cells located at the colony margin. Consequently, cells in the both regions undergo dissimilar fate [1,2]. More detailed understanding of principles involved in the formation and development of multicellular colonies requires new approaches allowing investigation of internal colony structure (at the level of individual cells) directly in situ, without cell removal from the microbial community. Recently, we succeeded in developing of a new approach enabling the monitoring of the presence and spatial localisation of fluorescently labelled proteins as well as of structures stained with specific fluorescent dyes within the whole Saccharomyces cerevisiae microcolonies, by use of two-photon excitation confocal microscopy [3]. Viewing the colonies from different angles allowed us to reconstruct a three-dimensional profile of the cells either producing specific GFP-tagged protein or possessing other specific properties visualised by staining with particular fluorescent dye. This technique allowed us even to monitor mutual organisation of microcolonies within a microcolony group.

We demonstrated that the production of Atolp-GFP (a GFP tagged variant of putative ammonium exporter essential for proper colony development) starts synchronously in cells located in the outermost surface layer of the microcolony. This layer exhibits a uniform thickness over the whole colony surface (of about 50 μm in the 3-days old microcolonies) and is independent of the colony diameter over a relatively wide range of 650-1000 μm. During later colony development, new fluorescent cell layer(s) become visible under the first layer, i.e. in areas more distant from the surface, again over the whole colony. These new layers are separated by thin layers (8-20 μm) of less fluorescent cells. The layers of cells producing Atolp-GFP are formed synchronously in all colonies developing nearby. GFP fluorescence localises predominantly to the plasma membrane and during later colony developmental phases also to vacuoles after degradation of GFP-tagged Atolp protein.

The new approach enabled us to uncover skin-like protective cell layer covering the whole microcolony. This “skin” is formed by living cells tightly joined via thick cell walls, probably connected by surface proteins. This layer protects the colony population against environmental impacts, for example, against impact of 50 % ethanol. Ethanol, when applied on the whole colony, affects moderately only plasma membranes of skin cells, while the cells inside the colony remain fully alive. On the contrary, when the same ethanol concentration is applied directly on internal cells, they are completely damaged.

The work was supported by GACR204/08/0718, IAA50020506, LC06063, AV0Z50200510 and MSM0021620858 and HHMI to Z.P.


Keywords: Yeast cell imaging in deep microcolony layers; skin-like cell structure; yeast differentiation.
Studies on the expression levels of the carotenogenic enzymes in the microalga Chlamydomonas reinhardtii

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Although microalgae and higher plants need light for photosynthesis, if the light input overcome the plant capacity to utilize it in the photosynthesis, the excess of light can cause over-reduction of the electron carriers and production of reactive oxygen species that will damage the cells. Among the mechanisms used in algae and plants to reduce the amount of energy that reaches the photosynthetical reaction centers, energy dissipation as heat is probably the most important short-term response. Thermal dissipation usually refereed as energy dependent quenching or feedback de-excitation is induced by a low pH in the thylakoid lumen, that occurs during illumination with excess light. Low pH is involved in the activation of the enzyme violaxanthin de-epoxidase, which catalyses the removal of epoxy groups from violaxanthin, to synthesize the de-epoxidised xanthophyll zeaxanthin via the intermediate antheraxanthin, these xanthophylls constitute the xanthophyll cycle.

Fig 1. Schematic representation of the xanthophyll cycle.

The expression level of the genes encoding the two first carotenogenic enzymes fitoene synthase and fitoene desaturase, and those encoding the main enzymes involved in the synthesis of xanthophylls, carotene hydroxylase and zeaxanthin epoxidase, has been analysed by quantitative real time PCR. Chlamydomonas reinhardtii pre-grown in the dark cells were submitted to light of increasing intensities (darkness, 150 and 800μEm−2s−1) and the concentration of mRNA corresponding to the indicated genes was determined and normalized to the concentration of Cblp mRNA. This gene encodes a homologous protein to the β subunit of a heterotrimeric G protein and has been previously shown to stay at constant expression level under different irradiances (Im and Grossman, 2001). The evolution of the expression of the chosen carotenogenic genes at the different irradiances has been correlated with the intracellular levels of the xanthophyll cycle components violaxanthin, antheraxanthin and zeaxanthin. After 3 h of exposure to high light (800 μEm−2s−1), about 20% of the total violaxanthin has been converted into zeaxanthin. This conversion increases in parallel with the light intensity and does not depend significantly on de novo synthesis of carotenoids, as stated in the presence of norflurazon.

References


Keywords: carotenoids; microalgae; Chlamydomonas reinhardtii.

Acknowledgements: We thank the Spanish Ministry of Education for financial support (AGL2007-65303-C02)

The effects of a calpain inhibitor upon human and plant trypanosomatids life cycles

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Peptidases of microbial pathogens have attracted the attention because of their roles in pathogenesis, which may lead to the design of chemotherapeutic agents. In this context, calpain inhibitors appear an interesting alternative. Calpains are calcium-dependent cysteine peptidases that are involved in crucial cellular functions such as cytoskeletal rearrangements and activation of various receptors and pro-enzymes. In this work, we aimed to explore the effects of the calpain inhibitor III (MDL28170) and to detect calpain-like molecules (CALPs) in Trypanosoma cruzi, Leishmania amazonensis and Phytomonas serpens. MDL28170 promoted a powerful reduction on the growth rate after 48 h, and the IC50 values were calculated to be 32 μM, 19 μM and 38 μM, respectively. This inhibitor promoted an increase in the cellular volume, but not cell lysis, resulting in a static effect upon Trypanosoma cruzi and Phytomonas serpens. On the other hand, a leishmanicidal effect was verified. Trypanosomatid CALPs presented a strong cross-reactivity with anti-Drosophila melanogaster calpain and, for T. cruzi, with anti-cytoskeleton-associated protein from Trypanosoma brucei antibodies, and labeling was found on the cell surface but mainly intracellularly. Furthermore, an 80-kDa reactive protein was detected by Western blotting assays. No significative cross-reactivity was found with anti-human brain calpain antibody. The expression of CALPs was decreased in human trypanosomatids kept for long periods in axenic cultures in comparison to strains recently isolated from mice, as well as in MDL28170-treated cells, the latter being paralleled by an increased expression of classical peptidases, such as leishmanial gp63 and trypanosomal cruzipain. Different levels of CALPs expression were also detected in distinct T. cruzi phylogenetic lineages, like Y strain (lineage TC1), Dm28c (TCII) and INPA6147 strain (Z3 zymodeme). These results may contribute for the investigation of the functions of CALPs in trypanosomatids and add new in vitro insights into the exploitation of calpain inhibitors in treating parasitic infections.

Keywords: Peptidase; Calpain; Inhibitor; Trypanosomatidae.
The Genotoxicity of Three Synthetic Pesticides: Chlorpyrifos, Cypermethrin and Their Mixture Chlorcyrin in Aspergillus terreus.

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In attempt to investigate the genotoxicity and the mode of action of the organophosphorous pesticide; Chlorpyrifos, the pyrethroid pesticide Cypermethrin and their mixture Chlorcyrin, the conidial spores of Aspergillus terreus were treated by five deferent concentrations (including the field concentration, 1ml/L) of each pesticide individually as follows:

Conidia were treated with 0.2, 0.4, 0.6, 0.8 and 1ml/L of each pesticide individually. Survival and mutation frequencies were calculated to find out the optimal dose for induction of mutation (by scoring the auxotrophs if possible).

As a result, it was found in each experiment that, with increasing of pesticide concentration and exposure time, a decrease in survival percentage and an increase up to a certain limit in mutation frequency were always observed. The optimal dose for inducing mutation by each of the pesticide used, was found to be 0.8ml/L.

Keywords: Genotoxicity, Mutagenesis, Chlorcyrin.

Thermoacidophilic archaea of Acidilobales ord. nov.: metabolic properties based on genomic data.

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3A new order of thermoacidophilic archaea Acidilobales which includes Acidilobaceae fam. nov. and Caldisphaeraceae fam. nov. was proposed based on the separate position of the Acidilobus – Caldisphaera group in the 16S rRNA-based phylogenetic tree, presence common specific signatures of in 16S rRNA, and common phenotypic properties distinguishing the representatives of this group from Desulfurococcales and other Crenarchaeota (Prokofeva et al., 2009). Members of Acidilobales order are anaerobic extremely thermophilic or hyperthermophilic acidophilic organotrophs with coccoid cells inhabiting terrestrial acidic hot springs.

To get insight into the metabolism and anaerobic thermoacidophilic survival strategy of these crenarchaeons, we have determined and annotated the complete 1496453-base genome of Acidilobus saccharovorans (Mardanov et al., 2009). A total of 1499 protein-coding genes have been identified, of which 246 are exclusive to A. saccharovorans. Genomic data reveal pathways for utilization of organic substrates and mechanisms of adaptation to hot acidic habitats. Utilization of polymeric carbohydrates and proteins involves the function of numerous encoded hydrolytic enzymes. Further oxidation of monomers proceeds in the modified Embden-Meyerhof and Entner-Doudoroff pathways followed by oxidative tricarboxylic acid cycle. The electron transfer chain is branched with two sites of proton translocation and is linked to the reduction of elemental sulfur and thiosulfate. Adaptation to acidic environment is reflected by high ratio of secondary over ATP-dependent primary transporters, the suggested reversibility of \( H^+\)-ATP synthase, and the function of the encoded \( H^+\)-translocating pyrophosphatase.

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Mardanov et al./Genome sequence of crenarchaeon Acidilobus saccharovorans reveals the mechanisms of adaptation to anaerobic thermoacidophilic lifestyle. 2009. Manuscript ready for publication.
Thymol affects expression of dnaK, groEL, htpG and tf genes of Salmonella enterica serovar Thompson

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Thymol is a natural antimicrobial and a component of some essential oils. Its inhibitory effect on the growth of different microorganisms is well known, while less known is its mechanism of action towards microbial cells. The response of bacteria grown in presence of thymol has not been satisfactorily investigated although it is known that a modification of membrane fatty acids composition is caused. However, it remains to be assessed whether the modifications in membrane composition really lead to an increased resistance or are just part of a general adaptive response. It is not known whether other mechanisms, mediated by the activity of stress associated proteins, are involved in the adaptation to thymol. To address this question four different proteins, classified as chaperone proteins, have been chosen to monitor their expression during different stage of the growth of a target microorganism. Salmonellae are significant not only as an ongoing threat to worldwide public health, but also as a model system for the study of fundamental mechanisms of bacterial pathogenesis, that is why it has been selected as target microorganism in this study. The four different proteins chosen were DnaK, GroEL, Trigger factor (Tig) and HptG, the expression of these proteins is commonly enhanced by the exposition of the microbial cells to stress conditions as chemical, thermal or oxidative factors. The growth of Salmonella enterica serovar Thompson was monitored in standard conditions and under the different stress factors, presence of thymol, NaCl and growth at 11°C. The expression of dnaK, groEL, tf and htpG genes was monitored by real time PCR after RNA extraction from cultures at different time of growth. It is possible to assert that the presence of thymol, as single stress factor, determined an over expression of all the genes, from 0.07 fold of the GroEL to 323.93 fold of the same protein, compared to control. Interestingly, the combination of thymol with the other stress factors do not lead to an over expression higher than the one registered with the thymol alone. On the other hand, it has been observed that the association of the thymol with the other stress factors led to an over expression lower than the one observed when the strain was exposed to NaCl or grown at 11°C, without thymol. The results indicate that the thymol induces a significant expression of stress related proteins. This over expression is surprisingly higher than the one related to the stress determined by NaCl and by low temperature, but strangely lower when the thymol is combined with the other stress factors. The regulation of the expression of these proteins, and certainly not only these, can be the basis of the adaptation of the cells to this antimicrobial compound. These results provide interesting information to any studies bridging the gap between mechanisms evaluated at the molecular level and observations at the organism level. A better understanding of the interactions between natural antimicrobials and cell target molecules is going to be fundamental to work out the best environmental conditions to be used to ensure an effective antimicrobial activity.

Keywords Thymol; Salmonella; Gene expression; Stress proteins;

Toxicity differences between Cr(VI) species in strain Ochrobactrum tritici 5bv11

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Abstract
Studies of Cr(VI) toxicity are generally performed using chromate salts in solution, both when studying the effects on prokaryotes and eukaryotes. Some studies on human carcinogenesis and toxicology on bacteria were done using dichromate, but comparison with chromate was never reported before, and dichromate existence was never taken into consideration and usually overlooked.

This paper studied comparatively the effect of dichromate and chromate on the physiology of O. tritici strain 5bv11, a highly Cr(VI)-resistant and reducing microorganism. This study demonstrated that the addition of chromate or dichromate sodium salts to growth medium at neutral pH ended up in two different solutions with a different balance of chemical species. Cr(VI) was toxic to O. tritici strain 5bv11, as clearly shown on growth, reduction, respiration, glucose uptake assays and by comparing cell morphology. Moreover, the addition of sodium dichromate was always more toxic to cells when compared to chromate and achieved a higher inhibition of every parameter studied.

The toxicity differences between the two Cr(VI) oxyanions indicate the possibility of a different impact of Cr(VI) contamination on the environment. This may be of major importance, considering the slight acidity of most of the arable lands which favours the presence of dichromate, the more toxic species.

Keywords Cr(VI); Ochrobactrum tritici; Toxicity; Physiology; Microorganisms;
Transcriptional regulation of ADE2 and PUT2 genes in *Kluyveromyces lactis*.

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The existence of ORFs with high homologies in those genes involved in the amino acids biosynthetic pathways points out that is possible to consider the existence of the same metabolic routes and regulation system. The ADE2 gene from *S. cerevisiae* is regulated by 2 mechanisms: the General Control through the transcriptional factor Gcn4p, and Basal Control through the factors Bas1p and Bas2p in absence of Gcn4p, which are characteristic of genes involved in the amino acids biosynthetic pathways (1). In *K. lactis* all the genes involved in these metabolic pathways have not been cloned yet, but those already characterized like *KlHIS4*, *KlTRP1*, *KlLEU2*, have shown that even they have the consensus sequences for these factors, the transcription is not activated in absence of aminoacids in the medium (Gcn4p dependent), nor in the absence of Adenine neither in the presence of phosphates (Bas1p and Bas2p dependent) like take place in *S. cerevisiae*. In this work, the analysis of the *KIADE2* promoter region have not shown the consensus sequences for these factors, and also have been confirmed by Northern analysis that repression by adenine described for ADE2 gene in *S. cerevisiae* have not take place in the same conditions.

*Saccharomyces cerevisiae* is able to use a wide variety of nitrogen sources for growth. In order to select the best out of a large diversity of available nitrogen sources, the yeast has developed molecular mechanisms. Proline is an important forerunner in protein biosynthesis but also plays an important role in other processes such as: cell protector under osmotic stress, radical acceptor, provisional nitrogen storage, etc. *Kluyveromyces lactis* can grow on proline as sole source of nitrogen. In the *K. lactis* genome, it has been reported the presence of homologous sequences to those *PUT1*, *PUT2* (this work), *PUT3*, *PUT4* and *GAP1* genes from *S. cerevisiae*, among others, implicated in this nitrogen metabolism system, indicating that the proline utilization in *K. lactis* is similar to that described in *S. cerevisiae* (3). This fact was confirmed with Northern analysis where we can postulate that *K. lactis* respond to induction by proline in the same way.

Thus, we report the isolation and characterization of ADE2 and PUT2 genes from *Kluyveromyces lactis*, including their protein structure, flanking sequence regions, and transcriptional gene regulation by different nutrients in the medium.

Keywords: yeast, transcriptional regulation, amino acid pathway, *Kluyveromyces lactis*.

References:


Acknowledgements: Supported by grant PGIDIT06PXIB103086PR from Xunta de Galicia (Spain).

Vital staining of yeast acidified vacuoles using neutral red

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Neutral red is a vital dye widely used in plants and animals histology. It is commonly used for the staining of lysosomes, the Golgi apparatus, or the Nissl granules in neurons. In addition, it has also been used as counterstain in combination with other dyes (i. e. with Janus Green B it is used to stain embrional tissues and supravital staining of blood). Besides its dye properties, neutral red is a pH indicator as well, changing from red to yellow in the range of pH 6.8 to pH8.0.

The vacuole of *Saccharomyces cerevisiae* is a lysosome-like organelle which plays an important role in its physiology. It maintains an acidic constant pH due to the presence of a V-ATPase in its membrane. The ATPase consists of 14 different protein subunits. Deletion of each of the corresponding genes results in vacuole acidification defective mutants named as vma.

Besides its similarity to the lysosomes, there are no methods reported to stain yeast vacuoles using neutral red. In this work we describe a group of experiments designed to set up a reproducible and reliable method for in vivo vacuole staining in *Saccharomyces cerevisiae*. Since neutral red tends to accumulate in acidic compartments, we also set up the conditions to use this staining in vacuole acidification assays. The wild type strain and some representative vacuole acidification defective mutants were used (vma2delta, vma3delta, sre/delta, sph1/delta). The advantages for rapid and easy vacuole acidification assays are described. Moreover, the fact that the neutral red only accumulated in vacuoles when the extracellular pH was higher than the vacuolar pH, lead us to fix the conditions to use the data of neutral red accumulation in the vacuole, as an indirect measure of the luminal vacuolar pH, when the cells are maintained in extracellular solutions buffered at different pHs. In addition, we have compared the usage of neutral red with the use of quinacrine, a fluorescent dye that can accumulate in acidic compartments too. Advantages and disadvantages of both methods in vacuole acidification assays are discussed.

Keywords: neutral red, *Saccharomyces cerevisiae*, vma, quinacrine, vacuolar acidification.
Volatile ammonia, the signaling molecule in different stages of multicellular yeast community development

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The way of life within yeast colony, an example of multicellular community of eukaryotic microorganism, differs significantly in many features from the life in shaken yeast culture. Life within a colony and survival of its population requires coordinated behaviour of individual cells in favour of the whole population. This has to be mediated by various signals. Ammonia functions as one of the signaling molecules influencing colony development in different stages. Volatile nature of this molecule enables long-distance signalling under natural conditions. Ammonia then also contributes to synchronising of development of group of colonies growing in the same territory [1].

We found, that both microcolonies (colonies arising from one cell) and giant colonies (colonies arising from cell suspension spotted on the agar) produce ammonia in pulses accompanied by alkalisation of the colony surroundings. These pulses are separated by acidification of the medium. Previous studies revealed that during second alkalization (7-9 day of development of giant Saccharomyces cerevisiae BY4742 colonies) ammonia induces alternative adaptive metabolism in cells of the colony and is important for differentiation of colony population and its survival [2,3].

We showed, that dependently on ammonia signalling, cells containing high amounts of proteins of adaptive metabolism (e.g. Pex1p, Cat1p, Cit2p, Lc1p, Ato1p, Ato2p and Ato3p) localise specifically to the colony margin. These cells are able to produce higher amounts of ammonia than central colonicular cells. On the contrary, central chronologically aged cells contain higher activities of stress-defence enzymes catalase Ctr1p and superoxide dismutases Sod1p and Sod2p, and they contain much higher activity of plasma membrane H+ATPase Pma1p than younger margin cells. This centre-margin differentiation predetermines the fate of colony cells; central cells undergo apoptotic-like dying and outer cells are fully capable to form healthy progeny.

Recent analysis of early development of microcolonies of Σ1278 derived strains, which are capable of dimorphic transition, suggested new function of ammonia during the first alkalization (6-24 hrs after colony inoculation) [4]. We discovered that early after new microcoenosis is originated from one cell, ammonia induces dimorphic transition resulting in oriented pseudohyphal cell expansion in the direction of ammonia source. This consequently leads to unification of adjacent microcolonies to one more numerous entity. Subsequently, unified microcolonies developed as one community. This mechanism helps to form more numerous community of cooperating cells, which possess higher capacity for protecting themselves against harmful environment. In addition, it prevents the possible competition of populations of small individual microcolonies for space and nutrients.

The work was supported by GACR204/08/2718, IAA500200506, LC531, AV0Z5020510 and MSM0021620858 and HHMI to Z.P.

Keywords: yeast colony differentiation and development; ammonia signalling; dimorphic transition

Vph1p, the vacuolar subunit of the V-ATPase in Saccharomyces cerevisiae, can compensate the lack of Stv1p, the Golgi subunit, to allow proper N-glycosylation of proteins

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In eukaryotic cells, the pH of the intracellular compartments is a carefully controlled parameter that affects many cellular processes. This control is done by a family of vacuolar ATPases in Saccharomyces cerevisiae is represented by a V-type ATPase. This enzyme consists of 13 subunits and requires 3 more polypeptides for its assembly. In S. cerevisiae, V-ATPase is located in the vacular membrane as well as in secretory pathway organelles like Golgi or endosome.

Deletion of any of the genes encoding V-ATPase subunits resulted in a non-functional V-ATPase and a characteristic vma phenotype. There are, however, two exceptions VPH1 and STV1 whose deletion do not produce a vma phenotype. They encode two isoforms of the V-ATPase "a" subunit, responsible for the enzyme location. Vph1p is mainly found in complexes isolated from the vacular membrane, while Stv1p is located in complexes of the Golgi membranes.

Previous studies have shown that Stv1p can compensate, at least partially, the lack of Vph1p in terms of vacuolar acidification. It has also been described that overexpression of STV1 in cells lacking VPH1, completely restored vacuolar acidification. In this work, we have checked several Golgi functions related to N-glycosylation in order to determine how the vacule located isoform Vph1p can compensate the lack of the Golgi located Stv1p in STV1 deleted strains. We checked for mannol phosphate transfer, outer chain elongation, and the addition of the terminal GlcNAc3Glc3Man9 (1,3) linked mannoses to N-linked oligosaccharides, in several strains: the "a" subunit deleted strains vph1delta and stv1delta; and the "common" subunits deleted strains vma2delta, vma3delta. In addition, we also checked the wild type and the double mutant vph1delta/stv1delta as controls.

The lack of the common subunits Vma2p or Vma3p resulted in a defective Golgi function, leading to a significant reduction of the mannosylphosphatase transfer and a slight reduction in the outer chain elongation and terminal mannosides addition. As expected, when Vph1p was not present, no effect on glycosylation was detected. However, unexpectedly, when the Golgi-specific subunit of the V-ATPase, Stv1p, was absent, N-glycosylation was not disturbed. This suggested that Vph1p subunit may compensate the lack of the Golgi Stv1p. To confirm this hypothesis, a double mutant lacking both subunits was constructed. In the double mutant, N-glycosylation processes were affected to the same extent as in vma mutants. These results reveal that the Golgi-specific Stv1p can be substituted by the vacuolespecific Vph1p, in stv1delta strains and suggest that the location determinants of the "a" subunit isoforms are not very strict, allowing them to travel to different locations, when needed.

Keywords: V-ATPase, Saccharomyces cerevisiae, STV1, VPH1, N-glycosylation, vma


Keywords: yeast colony differentiation and development; ammonia signalling; dimorphic transition
Alternative electron sinks of *Deinococcus geothermalis*

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*Deinococcus geothermalis* is known as an aerobic, highly resistant towards oxidative agents and tenacious biofilm forming bacterium causing biofouling in warm water industry. The strength and the compact biofilm structure is based on glycoconjugates present in the cell envelopes and numerous type IV pili like adhesion threads connecting cells to abiotic surfaces (steel or glass) and to neighbour cells.

Biofilm mode of growth may represent for the *Deinococcus* one way to minimize oxidative stress. Adhesion to electron conductive surfaces can offer a sink for the electrons discharged from the respiration chain. We investigated *D. geothermalis* ability to grow under microaerophilic and anaerobic conditions when supplied with alternative electron acceptors iron, manganese dioxide, nitrate and formate. The preliminary results showed that under microaerophilic conditions (O2 1%, with varying level of CO2) *D. geothermalis* grew as well in both oligotrophic and eutrophic media. Anaerobic growth with electron sinks other than oxygen rises the question if cell membrane or the biofilm e.g adhesion threads contain conductive material.

Acknowledgements: We acknowledge PhD fellowship from ABS graduate school, Academy of Finland, the Photobiomics grant and TEKES PolarKem-project.

**Keywords** *Deinococcus geothermalis*; biofilm; anaerobic; electrons

Anaerobic Baffled Tank (ABR) and Role of biofilm

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The overall objective of this study is the development of the most appropriate microbial culture for the wastewater treatment in developing countries. Two reactors constructed for the study. Firstly the reactor injected with low strength wastewater. The start-up of the reactor extended about 3 months. Once the biomass has been established, either as a granular particle or a floe, reactor operation is quite stable. Treatment of low strength wastewaters has been found to encourage the dominance of scavenging bacteria such as *Methanosaeta* in the ABR. Dilute wastewaters inherently provide a low mass transfer driving force between biomass and substrate, and subsequently biomass activities will be greatly reduced according to Monod kinetics.

The most significant advantage of the ABR is its ability to separate acidogenesis and methanogenesis longitudinally down the reactor, allowing the reactor to behave as a two-phase system without the associated control problems and high costs. Anaerobic systems can create better efficiency to very high organic loads removal and also the system efficiency will improve by temperature increasing and this system can be applied to treat domestic, small communities and industrial wastewaters in addition to leachate as a cost-effective system with high efficiency rate.

**Keywords**: ABR; wastewater; biofilm development
Antibacterial activity of quaternary ammonium monomers in solution and in non leaching coatings

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The retention of pathogen bacteria on contact surfaces increases the risk of cross-contamination and diseases transmission in industrial, hospital and domestic environments. Functional coatings containing biocides can endow antimicrobial properties to the surfaces in order to obtain free pathogen surfaces. The chemical bonding to the coating structure of monomers containing bioactive groups is a successful method to prevent the residual toxicity caused by a biocide release into the surroundings. In this study the antimicrobials activities of acrylic quaternary ammonium monomers (QAMs) in solution were investigated and compared with the antimicrobials activities of coatings obtained by insertion of QAMs into a urethane diacrylate structure by UV copolymerization. The effect of three dimethyl alkyl QAMs with alkyl chain of 2 (C2), 8 (C8) and 16 (C16) carbon atoms were investigated against Escherichia coli (E. coli), Staphylococcus aureus (S. aureus) and Listeria monocytogenes (L. monocytogenes). Figure 1 shows the reduction of the number of viable cells observed for cells in suspensions or attached on stainless steel surfaces after contact with QAMs solution or with coatings containing QAM mixities. The inhibitory effect differs with regard to the alkyl chain. The QAM with the shortest chain shows a limited effect towards cell suspension, while the C8 and C16 QAMs show high efficacy A survival decrease higher that the detection limit (> 6 log units) on L. monocytogenes. S.aureus is observed. However, the reduction grade was markedly lower on cells layered on stainless steel. Surprisingly, the C16 QAM bonded to the polymer structure lost the antibacterial activity. Results demonstrate that the C9 QAM modified coating could be a promising antimicrobial material but concentrations higher than 1% should be tested to achieve higher growth inhibition.

Antimicrobial and photocatalytic effect of silicate and silicone hygienic coatings

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Coatings with antimicrobial properties may play an important role to reduce the number of microbes and prevent microbial transmission. This is particularly important along a food chain, in water treatment plants as well as in pharmaceutical industry and hospitals. The conventional methods of disinfection with wiping are not effective in the longer term taking much time and use aggressive chemicals. Disinfection with hard ultraviolet C light is usually not satisfactory. Photocatalytic oxidation on surfaces coated with titanium dioxide might offer a possible alternative. Silicone and silicate paints based on photocatalytic active nanooxides were formulated and evaluated. Photocatalytic efficiency of coatings was evaluated as an absorbance change of organic dye Orange II solution. Antimicrobial properties of coatings were evaluated using agar plate methods and Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, Penicillium chrysogenum and Aspergillus niger suspension as a test microorganisms. Membranes impregnated with tested coatings were deposited on the surface of agar. Then the plates were inoculated by a suspension of test microorganisms. The growth/no growth pattern of microorganisms were determined after incubation under optimal conditions for test microbes. The antimicrobial activity of coatings was evaluated by measuring the zone of inhibition against test microorganisms. The coatings contained nanoparticles of titanium dioxide and zinc oxide showed the antimicrobial activity against test microorganisms. Whereas coating with zinc oxides nanoparticles successfully inhibited the growth of both bacteria and fungi, the photocatalytic nanoparticles of titanium dioxide inhibited bacteria but not fungi (weak inhibition has been observed).

Acknowledgement This study was supported by the Ministry of Education, Youth and Sports of the Czech Republic. Project No. 0021627502 and by GACR No. 203/08/1336, as well as project No. MPO FT-TA4/064.

Keywords hygienic coatings, antimicrobial effect
Biocides tolerance and architecture of opportunistic pathogens biofilms using the Calgary device

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Controlling the microbiological quality of surface is nowadays a major issue in food or medicine. Currently, and whatever the sector involved, treatment for cleaning / disinfection are undertaken regularly to ensure the hygiene of surfaces. The regulatory tests used to evaluate the antimicrobial activity of disinfecting agents are based on the use of cells in suspension or deposited and dried. However, in industry or in medical environment, microorganisms are usually found as biofilms (complex adherent cell included in a matrix of organic polymers) and are very different from their planktonic counterparts. This state of "community" generates resistance of microbial cells to the activity of disinfectants and thus increases the persistence of pathogenic microorganisms on the food chain. While the precise mechanisms underlying this resistance are still poorly understood, it appears as a multifactorial process primarily related to physiological and structural characteristics of the biofilm. Various factors such as the limited penetration of antimicrobial agents in the matrix, the physiological state of cells in the bulk of the biofilm or the expression of biofilm-specific phenotypes are beginning to be more clearly identified. In this context, we sought to identify structures and components that are involved in the resistance of these communities to the action of antimicrobial agents in three species frequently encountered in industrial and medical environments (Bacillus subtilis, Pseudomonas aeruginosa and Staphylococcus aureus).

Using the Calgary biofilm device, the susceptibility of 24h-biofilms of two strains of each species were evaluated for three disinfectants with different modes of action (peracetic acid, benzalkonium chloride and O-phthalaldehyde).

Results confirmed the greater resistance of some biofilms to disinfectants, as observed for P. aeruginosa with benzalkonium chloride. The structural study of biofilms by confocal laser microscopy scanning (CLSM) associated with the use of specific fluorescent markers (live/dead stain, lectins, metabolic activity tracers...) allowed us to characterize the heterogeneity of the architecture and composition of the biofilm. Relations have been found between the structural parameters of these biofilms (biovolume, thickness ...) and the resistance to antimicrobials tested. These results contribute to a better understanding of the relation between the structure and the properties of these biological communities. In order to guarantee the effectiveness of cleaning and disinfection treatments, the biofilm state should be considered in the establishment of new regulatory standards for assessing bactericidal activity of disinfectants.

Biofilm development during an anaerobic wastewater treatment process

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The anaerobic methods with immobilized biomass are discussed like effective and more appropriate for wastewater treatment in dairy industry because of high organic loading. In that aspect the development of an active and stable biofilm is one of the important prerequisites for achievement of high purification effectiveness of the treatment process. An anaerobic purification process in sequencing batch bioreactor with fixed biomass and gravel bed carrier was simulated in this study. The model wastewater, contained whey as a key pollutant was used. The simulated process was studied for a period from 282 days. The process was divided in three phases: i/ start-up and biofilm formation; ii/ transition from batch to semi-continuous regime; iii/ functioning of stable biofilm. The biofilm development and microbial restructuring during these three phases was studied according to the concept of r- and K-strategists. The microbial community in the water phase is in constant interactions with the immobilized microorganisms. Therefore the structure and functioning of the microbial communities of biofilm and free swimming biomass were investigated in parallel. To accelerate the critical phases of the initially formation of the biofilm as well as the process of its renewal and stabilization, the commercial preparations Laktazym and Bilikak were added.

Significant differences in distribution of r- and K-strategists were found depending on the phase of the process and variation of the technological parameters. During the initial biofilm formation in the water dominated slow growing microorganisms but on the inert material dominated fast growing heterotrophs. The obtained results showed that biofilm development is a result of a primary succession – in start-up dominated microorganisms with r-characteristics while during stable biofilm formation dominated microorganisms with K-characteristics. The stable and mature biofilm possessed high biodegradation activity nevertheless that in its structure dominated the slow growing bacteria.

Keywords: microbial community, heterotrophic biofilm, dairy wastewater
Biofilm formation by algae on sandstone monuments and their inhibition: A case study of Agra (India)

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Algae form thick biofilm on sandstone. The analysis of biofilm on stone showed a marked softening of the substratum and the progressive deepening of the biological growth in the layers beneath the surface that was due to the mobilization of elements and to enhanced water retention by polysaccharide sheaths. The species of algal biofilm produce and secrete a variety of metabolites which directly dissolve the stone compounds or increase their solubility. The influence of algal biofilm was studied on three sandstone monuments of Agra (India) viz. Sikandara, Christian Cemetery and Kailash temple. The study of diversity of algae showed dominance of Cyanobacteria members followed by Chlorophyceae and Bacillariophyceae members. The experiment approach included (a) SEM study of biodeteriorated sandstone (b) inclusion of See Spray™, H2O2, Pursue™ and Benzalkonium chloride in culture medium to visualize the in-vitro intervention of algal growth (c) estimation of Chlorophyll of treated algae (d) estimation of protein by Bradford method and SDS-PAGE (e) pH analysis and (f) vitality test.

Research revealed that quaternary ammonium compound – Pursue™ and Benzalkonium chloride (BKC) were appropriate algicide since they affect the cell membrane and chloroplasts. H2O2 couldn’t destroy the algal growth because of the presence of H2O2 scavenging system in Cyanobacteria while See Spray™ could inhibit the algal growth temporarily.

Key words – algae; sandstone; chlorophyll estimation; algicide; Hydrogen peroxide; quaternary ammonium compounds

Biofilm formation by kefir micro-organisms

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Kefir grains are dairy starters made of lactic acid bacteria, yeasts and, sometimes, acetic bacteria embedded in a polysaccharidic matrix produced by the micro-organisms. Grains look like cauliflower florets with a firm gummy consistency. Kefir grains form spontaneously in the Caucasus but an in vitro grain formation has never been reported. The aim of this work was to identify the pathway that may lead to an in vitro grain constitution by characterising the aggregation behaviour of kefir micro-organisms. Complete kefir microbial consortia, extracted from grains (KJ), were therefore incubated at 22 °C in different culture media and atmospheric conditions during 2 weeks. Culture media used were synthetic broths, regular milk or milk enriched with constituents known to enhance, directly or indirectly, the microbial polysaccharide production or the matrix firmness (yeasts extract, glucose or ethanol). The experiment was conducted twice at different time, with distinct microbial extracts. At the end of the incubation, the microbial consortia developed structures under some culture conditions: in the M17, MR5.5-4, Rogosa-CW and KPL broth incubated in anaerobic atmosphere, in the MR5.5-4 broth incubated in aerobic atmosphere and, once, in milk enriched with yeast extract. The structures developed in synthetic broth looked like frail biofilms (in M17, MR5.5-4 and Rogosa-CW) or fluffy heaps (in KPL) with poor consistence and elasticity. The structure developed in milk enriched with yeast extract looked like a sticky biofilm that resembled to mastic when kneaded between fingers. Microbial origin of this latter sticky biofilm was confirmed by microscopic observation under a magnification of 1000X that revealed plenty of lactobacilli and yeasts. As this microbial structure, that appeared to be the closest in consistence to kefir grains, was observed only once, the experiment in milk enriched with yeast extract was repeated 12 times: the sticky biofilm formed once again. The uncertainty trait of this event was attributed to the variability of the KJ grain microbial abundance [1]. To confirm this hypothesis and to bypass this difficulty for further examinations, the experiment was achieved with the kefir micro-organisms isolated in standardized pure cultures. By this way, sticky biofilm formation became a reproducible event as it occurred at each of the two assays performed. In conclusion, kefir micro-organisms formed structures in vitro but none of them appeared to be grains; they differed from grains in consistence and in shape. The one closest to grains in consistence was obtained in milk enriched with yeast extract.


Keywords: kefir, dairy starter, biofilm
Biofilm Formation of *Listeria monocytogenes* on Various Surfaces

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**Introduction & Objective:** *Listeria monocytogenes* is considered as a ubiquitous foodborne pathogen which can lead to serious infections, especially in newborns, elderly, pregnant, and immunocompromised people. The organism has been isolated from many foods and may cause meningitis, septicemia and abortion in pregnant women. Also, *L. monocytogenes* forms biofilms on many food contact surface materials and medical devices. Development of biofilms on many surfaces is a potential source of contamination of foods that may lead to spoilage or transmission of foodborne pathogens.

**Materials & Methods:** Biofilm formation of *L. monocytogenes* (RITCC 1293 serotype 4a) was investigated. Hydrophobicity of *L. monocytogenes* was measured by MATH method. Then biofilm formation of the organism was assessed at 2, 4, 8, 16 and 20 hours on stainless steel (type 304 no 2B), polyethylene and glass by drop plate method.

**Results:** Results indicated that *L. monocytogenes* with 85% of hydrophobicity formed biofilm on each of three surfaces. Biofilm formation on stainless steel surfaces was significantly more than other surfaces (p<0.05).

**Conclusion:** The ability of biofilm formation of *L. monocytogenes* on medical devices and food containers is very important as far as hygiene and disease outbreaks are concerned.

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Cell-surface hydrophobicity and corrosion characteristics of hydrocarbon degrading bacteria *Bacillus cereus* ACE2 and *Serratia marcescens* ACE4

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Two highly efficient hydrocarbon-degrading bacteria, *Bacillus cereus* ACE4 and *Serratia marcescens* ACE2, isolated from petroleum transporting pipelines, were identified by 16S rDNA gene analysis. This paper reports on the cell-surface hydrophobicity of these bacteria and its role in the corrosion of aluminium 2024 aeronautical alloy (AA 2024). The cell-surface hydrophobicities and emulsification index (E) of *B. cereus* ACE4 and *S. marcescens* ACE2 grown in hexadecane-containing medium are 86 %, E\textsubscript{24} 40\%, E\textsubscript{48} 66\%, E\textsubscript{72} 85\% and 60 %, E\textsubscript{24} 40\%, E\textsubscript{48} 66\%, E\textsubscript{72} 85\% respectively. The significant difference may be due to the efficiency of biosurfactant production which contributes to the increase in the cell surface hydrophobicity of the *B. cereus* ACE4 strain and enhanced bacterial adhesion on the AA 2024. Biocorrosion studies in minimal salt medium and surface analysis of the AA 2024 were carried out using weight loss method, and SEM-EDAX and FTIR respectively. Results showed that extracellular polysaccharides accumulate with the exposure time and revealed that biofilms are formed as micro-colonies, and subsequently causes pitting corrosion. The corrosion damage caused by *B. cereus* ACE4 is vigorous when compared to *S. marcescens* ACE2. The potential role of the high hydrophobicity bacterial isolates on biocorrosion of AA 2024 is discussed. This study highlights the importance of hydrophobic isolates in the biocorrosion of aircraft materials.

**Keywords:** Cell-surface hydrophobicity; Aluminium alloy 2024; *Bacillus cereus* ACE4; *Serratia marcescens* ACE2; Biocorrosion; SEM; IR spectroscopy
Characterization of corrosive bacterial consortia isolated from a cooling tower

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Microbial communities associated with biofilms are known to promote corrosion of cooling water pipelines. Knowledge of the microbial populations responsible for biocorrosion and the interactions of different microorganisms with metallic surfaces is required in order to implement efficient monitoring and control strategies. In this study, the analysis of the occurrence of corrosive bacterial community in water samples collected from cooling tower was performed using traditional cultivation techniques and identification based on 16S rRNA gene sequence. Seven aerobic bacterial species were identified: Pseudomonas putida ARTYP1, Pseudomonas aeruginosa ARTYP2, Massilia timonae ARTYP3, Massilia albidaflava ARTYP4, Pseudomonas mosselii ARTYP5, Massilia sp. ARTYP6 and Pseudomonas sp. ARTYP7. Although some of these species have commonly been observed and reported in biocorrosion studies, Oxalobacteraceae group members are identified for the first time in the cooling tower pipeline corrosion. The biocorrosion behaviour of copper metal by the new species ARTYP3 Massilia timonae was selected for further investigated using weight loss method, electrochemical techniques and surface analysis techniques (SEM and FTIR). Thin bacterial biofilms were observed and pitting corrosion was revealed after the removal of the biofilms. The potential role of each species in biofilm formation and the corrosion of steel is discussed.

Keywords: Cooling tower, Biocorrosion, Bacterial community, 16S rRNA gene analysis

Comparison of methods for detection of biofilm in Coagulase-Negative Staphylococci

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Coagulase-negative staphylococci (CNS) are most often associated with nosocomial infections, especially in premature and under weight newborns. The most important pathogenic factor of these microorganisms is the production of extracellular polysaccharide and the consequent formation of biofilm which facilitates their adherence to the surfaces of catheters and other medical devices. A total of 100 clinical samples of coagulase-negative staphylococci (CNS) isolated from infected medical devices received from the Neonatal Unit, University Hospital, Botucatu Medical School, including 50 isolated from catheter tips, 30 from blood cultures, and 20 collected from the nasal cavity of healthy subjects were investigated in order to evaluate the efficiency of three phenotypic methods of detection of biofilm formation, and also analyze the icaA, icaD and icaC genes, using the PCR method. The clinical isolates were screened by Tissue Culture Plate (TCP), Borosilicate Tube Method (TM), and Congo Red Agar (CRA) method. Of the 100 tested isolates, 82% were positive in the PCR method; in the TM, 82%; in the TCP assay, 81%; and 76% in CRA method. The method of adherence to the borosilicate test tube was the method that best correlated with the detection of the ica genes, showing better sensitivity and specificity when compared with the PCR technique. Our data indicates that the Tube Method is an accurate and reproducible method for screening and this technique can serve as a reliable tool for determining biofilm formation by clinical isolates of staphylococci, once it is also a fast, easy and low-cost method.

Production of biofilm by coagulase-negative staphylococci detected by the Tube method (TM). 1: sample not producing biofilm, 2 and 3: samples producing biofilm, 4: S. epidermidis ATCC 12228 (negative control), 5: S. xylosus ATCC 29979 (positive control).

Keywords: Biofilm, Coagulase-negative Staphylococci, Risk Factors, Infection, Phenotypic methods, PCR.
Confocal analysis of 60 biofilms structure using a microplate based high throughput method.

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Dominant role in the persistence of foodborne pathogens along the food chain is attributed to their ability of building cell communities with complex spatial structures and social self-protective mechanisms. They demonstrate pronounced adaptive resistance to antimicrobial stress in comparison with their planktonic counterparts. This may be related to physiological changes of surface-associated cells and/or reactivity of antimicrobials with the extracellular matrix. Both phenomena are directly related to the three-dimensional organisation of the biofilm. Understanding of structural architecture of cell communities could shed a light on their high antimicrobial biofilm resistance. In this context, our studies aim at qualitative and quantitative characterization of spatial biofilm structure for 60 foodborne pathogens using a high throughput confocal method. Six species were chosen for their implication in human infection (Listeria monocytogenes, Enterococcus faecalis, Staphylococcus aureus, Escherichia coli, Salmonella enterica and Pseudomonas aeruginosa) and 10 strains of each were selected to study intra-specific variability.

Biofilms were grown in Tryptone Soya Broth in 96-wells microplate during 24h. Confocal Laser Scanning Microscopy (CLSM) was used to record in-situ the 3D biofilm structure data. In order to visualize spatial structure and composition, biofilms were dyed with specific fluorescent markers. Recorded images were then processed to extract important biofilm structure parameters such as biovolume, thickness or roughness.

Under the described growth conditions, we observed a significant inter- and intra- species variability in biofilm formation and 3D architecture. Interspecies comparison of biofilm architecture has shown that E. faecalis and S. aureus strains formed compact biofilms with regular thickness whereas most P. aeruginosa strains produced the well-described mushroom-like mucoid structures. L. monocytogenes and S. enterica strains formed thin monolayer biofilms with small scattered clusters while E. coli produced rough biofilm. Biovolume heterogeneities were most pronounced for the biofilms grown from S. aureus, E. coli and P. aeruginosa species.

Our results have shown the diversity of biofilms architecture of 60 foodborne pathogens in static conditions. Specific three-dimensional structures which were identified can have a key role in (i) bacterial settlement (ii) tolerance to disinfection and (iii) persistence of pathogenic bacteria in the food chain. The combined use of microplate and confocal imaging proves to be a good alternative to the other high throughput techniques commonly used to study biofilm as it allows direct in-situ qualitative and quantitative characterization of these tri-dimensional biological structures.

Culturing phototrophic biofilms on surfaces: what determines biomass accumulation and species succession

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A range of microorganisms can be found living in close association to each other and attached to various surfaces forming structures known as biofilms. Natural aquatic biofilms are comprised of phototrophs (cyanobacteria and microalgae), and heterotrophs (bacteria, fungi and protozoa). They can form multilayered and highly structured communities maintained by a secreted exopolymeric matrix that also provides adhesion to surfaces. In order to enhance the understanding of the complex functioning of phototrophic biofilms it is necessary to investigate the effects that environmental conditions and key species have on their structure. Natural biofilms are difficult to investigate, because of the ambiguity of environmental conditions and the difficulty to maintain structural integrity during sampling. Laboratory microcosm experiments were thus performed where the main environmental variables could be controlled for biofilms grown on artificial substrata. Phototrophic biofilms, sampled seasonally from an Italian wastewater treatment plant (Congestri et al. 2006, Di Pippo et al. 2009) were cultivated in a specially-developed incubator where light, temperature and flow velocity could be closely controlled to determine their effect on changes in the phototrophic community structure and biomass accrual from colonization through to maturation.

During biofilm development the community composition differed greatly from the initial inocula. There was a marked decrease in taxon richness (about 50%) over the experimental period. In particular, there was a strong reduction of diatom diversity, Diadema confervacea was the only diatom to survive until the later stages. Coccolithophytes tended to attach first to the polycarbonate slides under high irradiance, while cyanobacteria colonised the initial stages of development of biofilms grown under low irradiance. Pseudomonas aeruginosa and S. aureus strains produced the thickest monolayer biofilms with small scattered clusters while E. coli produced rough biofilm. Biovolume heterogeneities were most pronounced for the biofilms grown from S. aureus, E. coli and P. aeruginosa species.

Our results have shown the diversity of biofilms architecture of 60 foodborne pathogens in static conditions. Specific three-dimensional structures which were identified can have a key role in (i) bacterial settlement (ii) tolerance to disinfection and (iii) persistence of pathogenic bacteria in the food chain. The combined use of microplate and confocal imaging proves to be a good alternative to the other high throughput techniques commonly used to study biofilm as it allows direct in-situ qualitative and quantitative characterization of these tri-dimensional biological structures.

Keywords phototrophic biofilms, biovolume, species composition, microcosm


Differences between clinical and food isolates of *Listeria monocytogenes* in biofilm formation

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Disruption of *Gluconacetobacter diazotrophicus* lsdA

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Pathogenic microorganisms as *Listeria monocytogenes* are known to form biofilms. The risk of food products contamination in food industry and the growth on medical devices, in the clinical environment, is a reason of concern.

The objectives of this work were to examine and compare biofilm formation by food and clinical isolates of *L. monocytogenes*.

A total of 376 *L. monocytogenes*, 258 isolated from various food products and 118 clinical isolates were investigated concerning their biofilm production capacity in 96-wells microtiter plates, at 4 °C during 5 days and at 37 °C during 24 h. The strains were classified as strong (OD_{630nm} > 0.220), moderate (0.110 ≤ OD_{630nm} < 0.220), weak (0.055 ≤ OD_{630nm} < 0.110) or no biofilm formers (OD_{630nm} ≤ 0.055) based upon the OD at 630 nm of bacterial films.

Statistically significant differences (P < 0.01) were found between biofilm formation at 4 °C or at 37 °C. At 4 °C, statistically significant differences between clinical and food isolates were observed (P < 0.01). Although being classified as weak producers, food isolates produced more biofilms than clinical isolates. Statistically significant differences (P < 0.01) between clinical and food isolates were also observed at 37 °C. However, at this temperature, clinical isolates produced more biofilms than food isolates; 4% and 0.4% of the clinical and food isolates, respectively, were classified as strong biofilm formers.

Biofilm production was demonstrated to differ between clinical and food isolates but these differences were temperature dependent.

This work was supported by FCT/FEDER Project PTDC/AGR-ALI/64662/2006.

Disruption of *Gluconacetobacter diazotrophicus* levansucrase encoding gene (lsdA) alters tolerance to abiotic stress, biofilm formation and sugarcane colonization.


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*Gluconacetobacter diazotrophicus* is a diazotrophic endophyte commonly associated to sugarcane and other host plants. Its levansucrase has been the subject of intensive research due to its ability to synthesize fructans of low degree of polymerization. However the biological role of levansucrases in nature is not well understood. For plant pathogenic bacteria, it has been suggested that levansynthesized by levansucrase, constitutes a physical barrier that isolates the bacterium, thus avoiding recognition by plant defense mechanisms. Levan is a highly hygroscopic, fluid polymer that could also contribute to tolerance to osmotic stress and biofilm formation. In this work, we evaluated the effect of the disruption of the levansucrase encoding gene (lsdA) from *G. diazotrophicus* in tolerance to osmotic stress, biofilm formation and colonization of sugarcane.

**Construction of lsdA defective strain.** The *G. diazotrophicus* levansucrase-deficient strain was constructed by interruption of an 1.4 kb with the *upf* cassette from pBSL46 that generated PSL46 *upf* plasmid. This was electroporated in *G. diazotrophicus* PAI 5 R, an spontaneous rifampicin resistant mutant, and transformants that recombined the disrupted locus were selected by phenotype, and PCR and Southern blot analysis. Mutant strain L-3 was selected for further studies. Disruption of the *lsdA* gene neither modified growth kinetics nor carbon source utilization.

**Tolerance to osmotic stress.** For tolerance to osmotic stress, effect of osmolytes is described at the indicated concentrations and its equivalent as osmotic pressure (π) is also indicated. When NaCl was used as an osmoyle, PAI 5 R and L-3 mutant strain grew similarly up to 25 mM NaCl (-0.609 MPa), but from 50 (-0.712 MPa) to 150 mM (-1.173 MPa) NaCl a reduction in tolerance was observed for the L-3 strain. At concentrations from 200 (-1.627 MPa) to 250 mM (-1.838 MPa) NaCl, both PAI 5 R and L-3 strain reduced their survival two orders of magnitude, but a difference in tolerance to NaCl was still observed among both strains, when 350 mM (-1.952 MPa) was used, none of the strains survived. Polyethylene glycol 400 (PEG 400) was used as a non-ionic osmolyte. Both strains tolerated up to 300 mM (-1.168 MPa); no growth was detected at 400 mM (-1.357 MPa). When sucrose was tested as an organic osmolyte, no difference on tolerance was observed between both strains, until a concentration of 676 mM (-3.607 MPa) was reached. L-3 strain was more susceptible at this concentration of sucrose. PAI 5 R and L-3 strains were also tested for dessication tolerance. Both strains were grown in liquid medium, cells were collected by filtration and the filters with the cells were incubated in Petri Dishes at 30°C and cell survival was estimated. By 18 and 48 h of incubation, a clear decrease in survival was observed for the L-3 strain as compared to PAI 5 R strain. By 72 h, PAI 5 R strain survived under dessication conditions but L-3 strain did not. After 144 h, none of the strains survived. The ability to form biofilms was evaluated in static cultures with Cristal Violet staining. L-3 strain showed a reduction of 30% in its ability to form biofilms when compared to PAI 5 R.

**Sugarcane colonization.** To evaluate the effect of levansucrase mutation in sugarcane colonization, PAI 5 R and L-3 strains were inoculated in sugarcane plants obtained by micropropragation and the quotient between the number of bacterial cells per tissue fresh weight and the number of inoculated bacterial cells was estimated. When the L-3 strain was inoculated, it showed lower numbers of bacterial cells associated to the roots or leaves of sugarcane plants at 4 h post-inoculation. At longer times (24 h post-inoculation) no difference was observed between PAI 5 R and L-3 strain.

**Concluding remarks.** Altogether these results suggest that the reduction in the ability of *G. diazotrophicus* levansucrase-deficient strain to colonize sugarcane is related in part with a reduction in its ability to adapt to the high osmotic pressure found in its sucrose-rich endophytic habitat, the sugarcane phloem and a reduction in its ability to form biofilms. This conclusion complements previous reports in which levan is related to the formation of a barrier that isolates the bacterium against the plant defense mechanisms.

**Keywords:** fructan, biofilm, osmotic stress.
Ecological approaches for dairy wastewater treatment

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The achievement of a good ecological status of receivers after discharge of waste or partially treated water from dairy industry requires harmonic interaction between water treatment technology and self-purification processes. The present research included two modules. First: an anaerobic treatment process for dairy wastewater in broadly spread sequencing batch bioreactor with fixed biomass was studied. As a source of active biological system was used specially treated and adapted activated sludge from Sofia Waste Water Treatment Plant. The immobilization of initially inoculated biomass, the addition of microbiological preparations, and its modification for increase of the ... to target contaminants were investigated as ecological approaches for the stimulation of water treatment process.

Second: self-purification processes in a water receiver for partially treated dairy wastewater were investigated. The functional role and restructuring of the microbial communities in the water, sediment water and sediments were studied.

The results showed that most important approaches for achievement of high effectiveness of wastewater treatment process were both - the adaptation and immobilization of biomass. In that aspect the data showed that biofilm in the bioreactors and in the river sediments plays the essential role in biodegradation of the target pollutants in comparison of the free swimming cells. The effect of the added preparations was lower. It thoroughly was related with low improvement of the rate of metabolism and functioning of the biological system mainly on enzyme level. The results confirmed the scientific hypothesis that as much the biodiversity of the biocenoses as much difficult is to incorporate allochthonic microorganisms in the community structure.

Keywords: adaptation, immobilization, biofilm

Effect of chromium on biofilm formation: bacterial biofilms in association with cyanobacterial strain.

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Three chromium resistant bacterial strains (SA1, SA2, SA3) and one cyanobacterial strain (SAP1) were isolated. The bacterial strains were isolated from two different biofilm samples collected from different sources. The cyanobacterial strain was isolated from the roots of hydrophytes. The bacterial strains SA1 and SA2 were twitch positive and SA3 was twitch negative. The isolated strains were checked for biofilm formation through microtitre plate assay. The biofilm formation was also checked on glass and coupons in presence and absence of Chromium (K2CrO4). The biofilms were observed through light and fluorescent microscopy and analysis was done through ----- . Biofilm was checked in combination of bacterial and cyanobacterial strains. The results showed that all the strains showed more attachment towards the glass. The combination of bacteria and cyanobacteria reveals that bacteria use cyanobacterial filaments as a substrate and attaches to it and form biofilms. Chromium reduction potential was also checked in biofilms of all the strains. Further the effect of biofilms on Triticum aestivum was checked. Results showed that the strains showed hydrophobic adherence towards the roots of the plants.
Effect of different synthetic and naturally occurring biocides on bacteria isolated from biofilms of dental unit water: their biofilm forming ability

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Dental unit waterlines (DUWL) are an integral part of dental surgery equipment, supplying water as a coolant, primarily for air turbine and ultrasonic scalers. DUWL when not in use remain connected to main water supply providing conditions for biofilm development within 8 hours. Bacteria shed from the biofilm can maintain and support massive number of planktonic organisms. Characteristically biofilm bacteria exhibit 3000 fold more resistance to surfactants, biocides and antibiotics than organisms floating freely in fluids. Biofilms on tubings within DUWL provide a reservoir of microorganisms and must be controlled. This study compared different biocides (synthetic biocides used in oral mouth wash and antibiotics) for their ability to reduce and eliminate the biofilm bacteria. Sodium dodecyl sulphate (SDS), Hydrogen peroxide (H2O2), Sodium hypochlorite (NaOCl), Phenol (Phe), Tween 20 (Tw 20), Ethylene dihydro tetra oxide (EDTA), Chlorhexidine gluconate (CHX) and Povidine iodine (Pi) were tested against DUWL biofilm bacteria. SDS, H2O2, Tw 20 and EDTA completely eliminate viable bacteria when applied singly, however, combined forms of these were found to be more effective in eliminating the biofilm bacteria. Some combinations effectively reduced the biofilm bacterial population. The most effective combination was of CHX with rest of the six biocides, although CHX gave the most consistent and sustained antimicrobial effect over time. In this study, the effects of eight biocides were monitored on DUWL biofilms individually and in combination by epifluorescence microscopy and total viable counts (TVC). Applying all the biocides simultaneously resulted in elimination of most bacteria. Combination of these biocides with cell wall constituents were also developed. Further the effect of these synthetic biocides (CHX) were compared with naturally occurring compounds in Neem plants, on biofilm formation by these isolates. Isolates were also compared for their ability of biofilm formation in the presence of naturally antibiotic producing strain of Streptomyces. Dental unit isolates growth was also compared with different antibiotics available in the market.

Effect of tyrosol in Candida species biofilm development

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Candida spp. are the most common agents of opportunistic myceses and are often associated with biofilms. The production of diffusible alcohol molecules allows the modulation of microbial physiological functions and probably offers a strategy for communication between Candida spp. Tyrosol has been shown to be a quorum-sensing signal in Candida albicans that is accumulated in planktonic and biofilm supernatants. Although tyrosol accelerates C. albicans germ tube formation under inducing conditions, the effect of tyrosol on biofilm formation is not clear. Additionally, to date it is not known whether tyrosol affects biofilm development of Non-Candida albicans Candida (NCAC) spp.

In this sense, this work aimed to examine the effect of a commercial formulation of tyrosol on different stages of NCAC spp. biofilm maturation, as an indicator of a role for this compound in biofilm development. Using a rapid and robust method (96-well plate model), the effect of tyrosol (10, 100 and 1000 μM) on C. dubliniensis CBS 7987, C. parapsilosis ATCC 22019 and C. tropicalis ATCC 750 biofilm formation was examined at different stages. C. albicans CECT 1472 was used as a control. Standardized cell suspensions (200 μl of 1 × 10^7 cells/ml in RPMI) were inoculated into microtitre plates and incubated for 5 and 48 h at 37°C, 130 rpm. At these time points the medium was removed and sessile cells were washed with ultrapure sterile water. Tyrosol was then added to Candida spp. sessile cells and the plates were incubated at 37°C for additional 24 h. Biofilm cells mitochondrial activity was evaluated by the XTT reduction assay and biofilm total biomass by crystal violet assay.

The results of these experiments showed that tyrosol did not exhibit major effects on C. albicans and C. dubliniensis biofilm development. However, tyrosol (10 and 100 μM) induced a significant reduction in the mitochondrial activity of C. parapsilosis sessile population and mature biofilms during subsequent development. The highest reduction levels of absorbance at 490 nm, with the control, were observed in mature biofilms treated for 24h with 10 and 100 μM tyrosol. Furthermore, addition of tyrosol (100 and 1000 μM) to C. tropicalis adhered cells population led to a significant decrease in biofilm cells mitochondrial activity during further development, as indicated by the lower levels of XTT readings compared with control biofilms. Under the conditions used in this study no changes were detected in total biomass of Candida spp. sessile cells treated with tyrosol.

These results show that tyrosol regulates C. parapsilosis and C. tropicalis biofilm cells mitochondrial activity, suggesting a role of this alcohol in the communication between Candida spp.

Keywords Candida spp.; biofilm; tyrosol
Evaluation of chemical and physical disinfection of process water and the treatment of biofilms in a pilot plant

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In several industrial and non-industrial applications, cooling water is reused and circulated in heat exchanger circuits. Cooling towers, heat exchangers, and humidifiers are just a few examples in which water is reused. However, continuous water reuse leads to a decreasing water quality due to the growth of microorganisms, biofouling, and corrosion. Microbical contamination of process water and formation of biofilms on the surface of piping and heat exchangers frequently result in operational problems and health risks. To avoid such problems, an effective, cost-efficient, and environmentally-friendly water treatment is required. Nowadays, a variety of physical and chemical techniques are routinely used for water disinfection, including chlorination and ultraviolet light. However, these disinfection methods suffer from severe drawbacks. Moreover, the ecological aspects of the various disinfections techniques have rarely been taken into account in their evaluation.

In a recently started research project, different chemical and physical disinfection techniques (sodium hypochlorite, peracetic acid, hydrogen peroxide, chlorine dioxide, ultraviolet and ultrasound) are evaluated in their efficacy to inactivate free-living bacteria and treat biofilms. Many studies have already focused on disinfection using ultrasound, UV, or chlorination, but to study disinfection techniques in standardized conditions, mostly lab-scale experiments were carried out. In this study however, experiments are performed on a pilot plant system for a better simulation of conditions encountered in industrial environments.

The main focus of the project is to explore possible synergistic or additive effects between chemical disinfectants and other techniques. Such synergistic effects can ultimately result in a decrease of the amount of chemicals that are required for effective disinfection and treatment of biofilms. In a first set of experiments, the physical disinfection techniques and different concentrations of chemical disinfectants were evaluated individually for their potential to inactivate free-living bacteria and treat biofilms.

When the inactivation of free-living bacteria is examined, treatment with UV, ultrasound and the chemical disinfectants resulted in a significant decrease in the number of live bacteria. Application of UV and ultrasound resulted in a 2-3 log reduction of the number of free-living bacteria. The effect of the chemical disinfectants on the number of free-living bacteria depended on the applied dose. Based on the concentration of the chemical disinfectants that is required to eliminate the free-living bacteria completely, hypochlorite was the most effective disinfectant, followed by chlorine dioxide. Peracetic acid was slightly more effective than hydrogen peroxide.

When biofilm-associated bacteria were assessed, it was observed that the efficacy of UV and ultrasound were comparable. Both physical disinfection techniques caused approximately a 1.5 log reduction in the number of biofilm-associated bacteria, which is less effective than the efficacy to inactivate free-living bacteria. Regarding the chemical disinfectants, it was observed that the concentrations that enabled the complete inactivation of free-living bacteria, were not sufficient to completely remove the biofilm-associated bacteria. Consequently, higher doses of chemical disinfectant is required to effectively remove biofilms. Again, hypochlorite and chlorine dioxide appeared to be the most effective disinfectants.

However, it must be stated that chlorine-based disinfectants are also associated with problems such as the development of resistance to chlorination, discoloration and the production of unpleasant odors and flavors, or the production of toxic by-products. A reduction of the required hypochlorite concentration and hence a reduction of toxic by-products may be achieved by combining hypochlorite treatment with a physical disinfection technique.

In preliminary experiments it was already demonstrated that a combination of physical disinfection techniques and chemical disinfectants resulted in a synergistic effect in the efficacy to treat biofilms.

Keywords: disinfection; biofilm; UV; ultrasound; chlorination; peracetic acid; hydrogen peroxide; pilot plant
Impact of peptidoglycan modifications on bacterial virulence: functional analysis of two *Shigella flexneri* genes; *orf185* and *orf186*

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1Funded by FRIA


*Shigella flexneri* is a Gram-negative pathogenic bacterium that causes bacillary dysentery, a disease responsible for one million deaths per year in developing nations. After ingestion, *S. flexneri* travels to the colon where it induces macrophages apoptosis and invades epithelial cells. The required virulence genes are located on a large virulence plasmid (pWR100) encoding a type III secretion system (T3SS).

Here, we investigated the virulence function of two uncharacterised plasmidic genes *orf185* and *orf186*. Proteins sequence alignment reveals that the product of *orf185* gene belongs to the Carbohydrate esterase family and *orf186* encodes for a potential glycosyl transferase involved in Peptidoglycan (PGN) modification. Here, we knock out (KO) the 2 genes and studied the phenotype of generated mutants both *in vitro* and *in vivo*. We show for the first time that *S. flexneri* can form biofilm. The formation of the later was enhanced upon inactivation of genes *orf185* and *orf186*. We also show that mutation of *orf185* and *orf186* genes reduce the bacterial entry into HeLa cells.

All together, our results pointed to the important role played by *orf185* and *orf186* in the potential PGN modification which consequently permit bacteria persistence within the precarious intracellular environment. Future work will address the molecular mechanisms involving ORF185 and ORF186. The achievement of this work will exceed the framework of *Shigella* in regard to the high conservation of the studied genes among several other pathogenic bacteria.

Key words: *Shigella flexneri*, PGN, biofilm.

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In *vitro* activities of the minocycline and EDTA solutions (CATH-SAFE®) against microorganisms embbeded in biofilm on the surface of hemodialysis catheters

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Catheters have become essential in the management of critical-care patients, yet the inside of a catheter can act as a source of infection due to biofilms. The use of minocycline and ethylenediaminetetraacetic acid (M-EDTA) as a potent calcium, magnesium and iron chelating agent destroy bacteria and fungal cell membrane and disrupts biofilm, thus allowing the associated antibiotic to exert its action locally at a high concentration. This study aimed to verify the *in vitro* action of the minocycline and M-EDTA solutions (CATH-SAFE®) on the biofilm produced in double lumen hemodialysis catheter. Viable cell count and scanning electron microscopy (SEM) were the techniques used. For biofilm formation, the catheter was immersed in Mueller-Hinton broth previously inoculated with suspension of 1.0x10³ CFU.mL⁻¹ of *Staphylococcus aureus* and *Staphylococcus epidermidis*, and incubated at 37°C for 120 hours. Then, the catheter was removed and 3 ml of M-EDTA solution (CATH-SAFE®) - Minocycline hydrochloride 1mg and 30mg EDTA) instilled, remaining in the lumen of catheter for 72 hours. After this period, the solution was removed and the catheter segmented in 1 cm sections separated in hub, mean, tip. The segments were accessed to viable cell count and prepared for observation by SEM. The viable cells count of *S. aureus* without the application of M-EDTA solution (control) were: hub - 5.6x10⁴ CFU.mL⁻¹; mean - 5.6x10⁵ CFU.mL⁻¹; tip - 5.6x10⁶ CFU.mL⁻¹. After applying the M-EDTA solution viable cell count of *S. aureus* were: hub - 1.3x10⁴ CFU.mL⁻¹; mean - 2.6x10⁵ CFU.mL⁻¹; tip - 3.0x10⁶ CFU.mL⁻¹.

For *S. epidermidis*, counts of viable cells without the application of M-EDTA solution (control) were: hub - 3.7x10⁵ CFU.mL⁻¹; mean - 9.3x10⁵ CFU.mL⁻¹; tip - 2.2x10⁶ CFU.mL⁻¹. After application of M-EDTA solution, the viable cells count of *S. epidermidis* were: hub - 1.4x10⁶ CFU.mL⁻¹; mean - 5.6x10⁷ CFU.mL⁻¹; tip - 8.5x10⁷ CFU.mL⁻¹. According to data obtained, there was a reduction of *S. aureus* and *S. epidermidis* colonization approximately of 100-times after the 120 hours exposure to M-EDTA. SEM showed cocci groups adhered in the lumen of the catheter. The use of minocycline and M-EDTA solutions in catheters may prevent a risk of sepsis in hemodialysis patients due to decrease of biofilm formation.
Microbial cell surface hydrophobicity and surface energy obtained using the sessile droplet technique: identification of sources of inaccuracies due to the topography and chemical heterogeneity of microbial lawns.

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General Considerations. Microbial Hydrophobicity and surface energy are fundamental properties to study interfacial phenomena in which they are involved, such as their adhesion to surfaces. One of the main methodologies used to quantify these properties is the contact angle method, which lies in the measurement of equilibrium contact angles of liquid droplets resting on microbial cell (multi)layers. This same approach is usually used to quantify these properties for the surfaces the microbes are interacting with (e.g. biomaterials), which are commonly characterized in a polished state. Characterization of both microbial and materials surfaces allows theoretical models to be explored for the rationalization of the interaction process. This methodology is common in the investigation of initial microbial adhesion (thought to be of a reversible physico-chemical nature), the first step in the development of biofilms.

Motivation. Measurement of equilibrium (Young’s) contact angles assumes smooth and homogenous surfaces. These two premises are commonly verified for material surfaces before contact angles are measured. In the case of rough and heterogeneous surfaces, observed contact angle may largely differ from the Young’s contact angle, as both types of irregularities cause contact angle hysteresis. Very little is known however about surface topography (roughness) and heterogeneity of the microbial lawns used to characterize their properties.

Objectives. Unfortunately, microbial surfaces can not be polished! Therefore, our goal was the exploitation of atomic force microscopy (AFM) to quantify the surface topography of bacterial lawns, as well as its Phase Imaging (PI) mode to explore their physico-chemical homogeneity. The quantification of the level of non-ideality of these microbial surfaces will allow to ascertain the level of uncertainty in the determined cell surface properties, and therefore, in the predictions made by entering these values into available theoretical models.

Results. The relevant roughness parameter in relation to contact angles is Wenzel roughness factor, defined as the surface-to-projected area ratio, which can be accessed experimentally via AFM by simple triangulation. Values as high as 1.3 have been obtained (for comparison, values of about 1.005 are commonly found in polished materials), which means a very rough surface. This is comparable to the roughness displayed by polymers, and definitely roughness can not be neglected. On the other side, PI has identified the presence of a ultrathin (few nm) liquid-layer discontinuously covering the bacterial lawns, rendering them extremely heterogeneous. We propose that the origin of such a stable (non-evaporating) liquid-like layer lies in the deliquescent behavior of one the two components of the buffer used (KPi), K2HPO4, as discussed also in another of our presentations, which is able to absorb water from the environment and form a (liquid) dissolution at the surface.

Conclusions. Bacterial lawns commonly used for the determination of their hydrophobic properties have been analyzed using AFM. Results reveal a very rough and chemically heterogeneous surface, which compromises the use of the Young’s contact angle concept in this context. The potential inaccuracies in the measurement of the microbial cell surface properties using this method might be one of the key reasons of the lack of success of established colloidal models to analyze microbial adhesion and of the variability at inter- and intra-laboratory levels.
Microphytobenthic biofilms in the Cabras lagoon (Sardinia, Italy)

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The Cabras lagoon (western Sardinia, Italy) is a transitional system renowned for its naturalistic Ramsar Convention on Wetlands, Natuna 2000 network for EU Habitat directive and economical importance (e.g. artisanal fisheries). However, the lagoon is increasingly subjected to anthropogenic pressure due to massive nutrient loading, reduction of freshwater input from upland, modifications of the inlets and other man-made interventions which have reduced the water exchange with the adjacent Gulf of Oristano. Especially during the warm season, there is a tendency for hypoxic and anoxic conditions to occur in near-bottom waters. This may lead to dystrophic events, causing a major loss of the biological resources of the lagoon. To assess the ecological quality of the lagoon, numerical models have been developed to predict the evolution of both hydrological and ecological variables within the lagoon system under different meteorological forcing. In parallel, investigations of the physical and chemical characteristics of the sediments and the macrobenthic assemblages have shown a close link between the distribution of organic-C binding fine sediments, benthic macroinvertebrates, and the water residence times computed from the model [1].

In these systems, a detailed analysis of sediment dynamics are particularly important because the partitioning and transport of fine sediments can strongly influence the redistribution and accumulation of large amounts of organic matter and consequently the spatiotemporal distribution of benthic assemblages and the trophic status and functioning of the lagoon [2, 3]. Primary producers are considered to be an important source of organic matter to the Cabras lagoon [2]. Nevertheless, studies on the presence and distribution of microphytobenthic assemblages in the Cabras lagoon are still lacking although the importance of this highly variable, biological component has been shown in other organic-enriched coastal lagoons [4-6].

Benthic phototrophic primary producers may develop at the sediment surface in form of biofilms in which cyanobacteria and microalgae are embedded in a common exopolymeric matrix (EPS) [4, 7] that contributes to the cohesive properties of the fine sediment fraction [8].

To evaluate the microphytobenthos biomass surface sediment, six sample replicas were collected at three sites in the Cabras lagoon and chlorophyll a (Chl a) was extracted in 90% acetone and spectrophotometrically determined. Accessory photosynthetic pigments were also estimated to evaluate the proportion of the different phototrophic taxa in the communities. Samples were analysed by light and fluorescence microscopy to assess the spatial distribution and composition of microphytobenthic species in relation to sediment characteristics. In addition, Alician Blue cytochemical stain at pH 2.5 of EPS was used to ascertain the presence of colloidal and bound polysaccharides. Preliminary results showed microphytobenthic biomass highly variable in space between and within sampling sites with Chl a content values ranging between 4.1 and 107.4 μg g−1 dry wt. However, values of accessory pigments concentrations and their ratios to Chl a contents suggested the dominance of diatoms and the presence of cyanobacteria and green algae in most of the biofilm communities. The spatial variability was also confirmed by microscopy observations showing the heterogeneity of microphytobenthic species composition. Acidic colloidal polysaccharides were visualised with Alician Blue stain in diatom-dominated biofilms, while bound polysaccharides were prevalently found in the sheaths of the filamentous cyanobacteria.

Keywords: microphytobenthos, phototrophic biofilms, exopolymers, cyanobacteria, microalgae

References


Monoculture and mixed biofilms of Listeria monocytogenes and Pseudomonas fluorescens – evidences of antagonism and self-repression


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Listeria monocytogenes is a pathogen responsible for severe illness with high mortality rates. It is frequently found in different kinds of food processing environments and its capacity to develop biofilms is an issue of major concern on food safety. Although many studies have been made concerning adhesion and biofilm formation by mono-cultures of L. monocytogenes, it is known that the vast majority of microorganism in the natural environment and in the food processing environment occurs in multispecies biofilms. Several works have shown that association with strains belonging to other genus, as Pseudomonas, Flavobacterium and Staphylococcus, may increase or decrease L. monocytogenes attachment and biofilm formation, depending on the strain. In this work, both monoculture and mixed biofilms were performed in order to investigate how biofilm formation by different isolates of L. monocytogenes is affected in presence of Pseudomonas fluorescens. Moreover, taking into account the cases where an antagonism effect was found, different supernatants were collected and tested for their influence alone on biofilm formation by L. monocytogenes.

The L. monocytogenes strains assayed were CECT 4031 (collection strain), 747 and 994 (food isolates), 1559 (environmental isolate) and 1562 (clinical isolate). P. fluorescens strains used were ATCC 27663 (collection strain), and PFA7 (food isolate). Each L. monocytogenes strain was tested for monoculture and mixed culture biofilm formation with each one of the P. fluorescens strains. Assays were performed during three days in 96-well microtiter plates, at room temperature (22 ± 2°C) with shaking at 120 rpm. The medium used was BHI (replaced every 24 hours) and the biofilm biomass was assessed by the Crystal Violet method. Supernatants were obtained from overnight cultures and biofilms also grown at room temperature, which suspensions were collected, centrifuged and filtered through 0.2 μm filters. Results concerning monoculture and mixed biofilms formation revealed a significant decrease of biomass in biofilms formed by CECT 4031 and 1562 L. monocytogenes strains when in the presence of both P. fluorescens strains, with the strongest antagonist effect being found between 1562 and PFTA7 isolates. These two last isolates were then used to obtain supernatants to be applied during biofilm formation by CECT 4031 and 1562 L. monocytogenes strains, in order to infer about possible excreted metabolites that could influence biofilm formation. The results of this approach showed that PFTA7 +1562 mixed biofilm, 1562 overnight suspension and 1562 biofilm supernatants lead to a significant biomass decrease when applied on CECT 4031 biofilm formation. Although this does not help to explain the antagonism effect of PFTA7 upon this Listeria strain (which in turn may be associated with strictly intracellular quorum-sensing phenomena), it highlights an interesting repression within these two L. monocytogenes strains. On the other hand, 1562 biofilms suffered a significant decrease in biomass when formed in the presence of PFTA7 biofilm. PFTA7 +1562 biofilm and 1562 biofilm supernatants. These results are consistent with the possibility of an antagonism based on the activity of extracellular metabolites produced during biofilms growth and, once again, auto-repression phenomenon is pointed out, this time concerning only the 1562 strain.

In general, this work is in accordance with many other authors who found the influence of different bacterial genus on L. monocytogenes biofilm formation to be highly dependent on the strains. Moreover, it has shown that, at least in some cases, the antagonistic effect on mixed biofilms may be based on the action of metabolites that are produced during biofilm formation and secreted to the surrounding media. In order to support this hypothesis, further investigation is needed on supernatants composition and activity of their components. In addition, this work has also pointed out a very interesting auto-repression phenomenon, not only between different L. monocytogenes strains but also within the same strain. This shows how complex biofilm regulation can be and adds auto-repression as one more aspect to be considered when studying biofilm formation by this bacterium.

Keywords: Listeria monocytogenes; Pseudomonas fluorescens; mixed biofilms; antagonism; auto-repression
Motility and biofilm formation ability of isolated vs collection P. aeruginosa: effect of single and combined antimicrobial application

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Biofilms can be defined as communities of microorganisms attached to a surface. During biofilm development, sessile cells acquire physiological characteristics differentiating them from planktonic cells which include an increased resistance to antimicrobial treatment. Pseudomonas aeruginosa is an opportunistic pathogenic bacterium, considered as the normal bacterial flora of the pharynx, mucous membranes and skin, and is widely investigated for its high incidence in clinical environments and its ability to form strong biofilms. When this organism is isolated from clinical settings, efforts should be made to substantiate its clinical relevance often using a collection strain as control. The current treatment to eradicate P. aeruginosa favours the use of empirical antimicrobial combinations, balancing the potential for greater toxicity against the lower emergence of antimicrobial resistance and the greater killing that might be achieved by combination therapies acting synergistically. Studies showed that co-application of antimicrobial agents in some cases allowed improvement of biofilm destruction compared with single drug attack. With this work it is aimed to characterize and compare isolated and collection P. aeruginosa regarding its motility and biofilm formation ability and how this is affected by single or combined antimicrobial treatment. P. aeruginosa from collection (ATCC 10145) (PAC) and P. aeruginosa isolated (PAI) from a medical device (endoscope) were used as biofilm producers. Biofilms were formed for 24 h in 96-well plates, being then non- and treated with 1 mM benzalkonium chloride (BZK), 1 µg/ml of Ciprofloxacin (Cip), and a combination of both, for 30 min. BZK is applied as a clinical disinfectant and antiseptic in health care facilities and domestic households and Cip is an antibiotic used to treat urinary tract infections. Crystal Violet (CV) staining together with XTT, were used to assess total attached biomass and respiratory activity, respectively. To evaluate bacterial motility, swimming, swarming, and twitching assays were performed.

The data revealed that resulting PAI biofilms have less attached biomass and respiratory activity when compared to PAC biofilms. Also it was shown that alone none of the antimicrobial agents selected are effective on biomass clearance. BZK seems to favor biomass accumulation whereas Cip slightly affects only the PAI biofilm. Moreover, when combined, BZK and Cip seem to improve the detachment of both strains biofilms. As regards respiratory activity, when exposed to Cip attack only the PAC strain activity was affected. On the other hand, BZK have promoted the PAI biofilms activity decrease. Furthermore, the combined action of antimicrobial agents affects both biofilms activity. Regarding bacterial motility, results showed that the motility effects are mainly notorious in PAI, which have revealed larger diameters of the migration zones in all the assays. The flat colony suggests that these strain spread mostly by swimming motility.

This study allowed understanding that the combination of two antimicrobial agents might not be an advantage in what concerns to P. aeruginosa biofilm removal, and thus to surface disinfection. Comparing both strains, it can be concluded that PAI have less ability to attach the surfaces and to develop biofilm than PAC. Taking into account that PAI is constantly under stress conditions provided by the exposition on its natural environment, this isolated strain probably developed resistance mechanisms that may led to phenotypic changes at locomotive appendix level (flagella and pili). These cell surface structures where shown to play an important role in the early events of biofilm development in a wide variety of surfaces, namely by making possible the flagellum-mediated swimming movement and further P. aeruginosa surface contact.

Keywords: Biofilm; P. aeruginosa; Antimicrobial agents; Motility, Locomotive appendixes


On site monitoring of biofilm formation on quartz quarries using colorimetric techniques

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Quarries and other mining activities cause an important impact on the landscapes and the companies are obliged to restore the degraded areas. In the case of quartz exploitations the common techniques for reducing the visual impact, based on the re-vegetation of open-rock faces, are not efficient due to the characteristics of the mineral (chemically inert, very hard and with very low porosity and, consequently, with low capacity of water absorption). As an alternative technique, the induction of biofilms on quartz surfaces was evaluated.

In this work we present the results of the monitorization of biofilm development on quartz surfaces in the “Serrabal” quarry (Vedra, A Coruña, NW Spain), where a highly pure white quartz is extracted. Test areas from selected open-rock faces were inoculated with microorganisms isolated from biofilms that had grown spontaneously on quartz outcrops close to the mine, and water and different nutritional products were applied on the test areas in order to favour the development of biofilms. The biofilm microorganism composition was characterised by molecular biology techniques, which permitted to identify algae, cyanobacteria and bryophyte species.

Biofilm development was monitored on site by colour measurements, according to previous studies which demonstrated that colour measurements allow accurate quantification of colonization progress by coloured organisms directly on the field, even when the level of colonization is not detectable by the human eye (Prieto et al. 2002; Prieto et al. 2004). Colour measurements were carried out using an portable spectrophotometer and were expressed within the CIEL*a*b* colour space. ΔE*, total colour difference, was the parameter that best reflected the biofilm development and its physiological evolution along time. The evaluation of the chromatic parameters (a*, b*, and C*) and luminosity (L*) permitted to determine the beginning of biofilm development and also to evaluate its physiological evolution along time.

Keywords: on site monitoring, CIELAB colour measurements, biofilm induction.

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PAH degrading Bacterial community from the Sea Surface Microlayer in an estuarine system

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The sea surface microlayer (SML) is a thin biogenic film on the surface of the water bodies that physically bounds the ocean and the atmosphere. Due to its hydrophobic nature, important amounts of autochtonous and anthropogenic recalcitrant compounds such as polycyclic aromatic hydrocarbons (PAH), tend to concentrate in the SML, in relation to the UW. Although it is accept that such levels of contamination may activate the bacteria communities capable of degrading this compounds, little is known about their structure. The aim of this study is to compare the bacterioneuston and bacterioplankton communities in terms of abundance and structural diversity along a PAH contamination gradient in an estuarine system. The culturable fraction of PAH degrading bacteria from the SML was also characterized. Fluorescence In Situ Hybridization (FISH) was applied to study total microorganism abundance and quantification of Bacteria and γ-Proteobacteria specific groups. Denaturing Gradient Gel Electrophoresis (DGGE) was applied to access diversity of 16S rRNA gene fragments and Pseudomonas-specific gacA gene fragments. PAH degrading bacterial strains were isolated using naphthalene as a sole carbon source. PAH microorganisms abundance was similar between the two compartments of the water column. The relative abundance of the Bacteria domain was also similar between the SML and UW. The γ-Proteobacteria subclass showed the highest values at the estuarine sections where the concentration of low molecular weight PAH was highest. According to the DGGE profiles of 16S rRNA gene fragments, the overall bacterial diversity was similar in bacterioneuston and bacterioplankton communities. Pseudomonas-specific gacA gene fragments analyses suggest that diversity of this genus is higher in the SML. Pseudomonadaceae and Enterobacteriaceae families were the dominant PAH degraders in the culturable fraction of the bacterioneuston in a contaminated site from the estuarine system Ria de Aveiro.

Keywords sea surface microlayer; polycyclic aromatic hydrocarbons; bacterioneuston.

Phosphomonoesterase and phosphodiesterase activities of cultured phototrophic biofilms

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An increase in extracellular phosphatase activity has often been reported as response to phosphorus limitation in a wide range of organisms. An increase in activity indicates a potential to degrade organic matter is closely coupled with phosphorus (P) uptake. A study of phototrophic biofilms reconstructed of strains isolated from a wastewater treatment plant was undertaken and cultures started under diverse phosphorus regimes in a continuous flow incubator, P replete, P limited and organic P only. Artificial colorimetric substrates that produce a colorimetric product when hydrolysed were used to determine phosphomonoesterase (PMEase) and phosphodiesterase (PDEase) activities of the cultures over an 18 day incubation period. PMEase and PDEase activities of full biofilms, cells only, and medium were measured and compared with biofilm nitrogen (N) and P content, and biomass accumulation (dry weight).

Biofilm growth was higher under P repletion than the P limited and organic P treatment; growth under the latter two treatments was not significantly different. Under all treatments there was a decrease in cellular P during growth suggesting growth dilution of cellular P. As a function of dry weight, biofilm phosphorus content decreased whilst nitrogen was generally unchanged and resulted in large increases of the biofilm N:P indicative of stoichiometric P limitation. PMEase and PDEase were measurable on each occasion for all biofilms possibly a result of continual P requirement due to biofilm nutrient status (growth dilution of P or the increase in N:P) or the presence of constitutive activity. The P content of the organic P biofilm was high compared to the P replete biofilms and indicates the efficiency of phosphatase mediated P uptake, although this was aided to some degree by the slowing of growth to maintain cellular P levels. Sites of phosphatase activity were mainly cellular (PMEase, 59%; PDEase, 77% of the total activity) compared to the activity in the matrix. Activity was almost entirely restricted to the biofilm as phosphatases were not released into the medium. This suggests that biofilm P requirements are mostly met by recycling of biofilm derived organic material and interstitial sources. PDEase has been shown to be a secondary response to higher P limitation and this would adequately explain the increase of PDEase activity later in the incubation period when biofilm P content decreased to around 0.5% of the dry weight. The use of cultured biofilms was shown to be a useful tool in unravelling the complex processes involved in nutrient acquisition and utilization within these complex microbial communities.

Keywords Phosphomonoesterase, phosphodiesterase, biofilm, phototrophs, phosphorus limitation, nutrient status, N:P ratio
Plasmids, antibiotics and their influence on the formation of dynamic Escherichia coli biofilms

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Escherichia coli is commonly used as an indicator organism for food and water contamination and is frequently found in biofilms formed on piping systems. However, compared to the wealth of information concerning biofilm formation by other Gram negative bacteria, such as several members of the Pseudomonas genus, comprehensive information regarding the dynamic formation of E. coli biofilms is not yet available.

For certain Pseudomonas species it is known that biofilm-forming cells are more resistant to antibiotic treatment than their planktonic counterparts. However, these findings were made using cells that are naturally susceptible to these antimicrobial agents.

On this work we set out to investigate the influence of antibiotic addition on the biofilm-forming capacity of cells that have been transformed with a plasmid conferring resistance to kanamycin. Thus, we have compared the kinetics of biofilm formation using “empty” E. coli JM109(DE3) cells grown on a glucose containing medium (case 1) with the results obtained when these cells were previously transformed with the pET9a vector and the medium supplemented with 20 μg/mL of kanamycin (case 2). Kanamycin resistance genes have been extensively used as selective markers on several recombinant vectors such as pET9a (from Novagen). These genes encode bacterial aminoglycoside phosphotransferases that inactivate kanamycin by transferring one phosphate from ATP to a specific position on the antibiotic molecule.

In order to assess the influence of the plasmid and of the antibiotic presence on the biofilm-forming capacity of the cells we have used an experimental system that includes a recirculating tank, where planktonic cells grow, and a flow cell system for biofilm formation. This flow cell is composed by a poly(methyl methacrylate) semicircular duct with 10 apertures on its flat wall where several removable rectangular pieces (coupons) are placed. Sampling is performed by stopping the flow, removing the coupon of interest, replacing it with a sterilized one and starting the flow again.

When no antibiotic is added (case 1) planktonic cell culture reaches a much higher optical density than the one attained in case 2 (transformed cells and antibiotic added). Since the culture medium composition is the same on both cases we speculated that the metabolic burden of hosting a high-copy plasmid and/or the resource drain caused by the expression of the resistance gene might be responsible for this decreased cellular concentration. However, by analyzing the biofilm cells we see that E. coli JM109(DE3) grown without antibiotic (case 1) maintain a relatively constant cellular density of approximately 8.20E6 cells/cm³ throughout the experience whereas when transformed cells are used and the antibiotic is added (case 2) the number of attached cells increases along the experimental time reaching a maximum value which is much higher than the average value attained in case 1. Additionally, comparing the biofilm wet weight and thickness we have observed that when transformed cells are used (case 2) thicker and heavier biofilm samples are obtained. Thus, it seems that the primary effects of either the expression of antibiotic resistance genes and/or the maintenance of a high-copy plasmid are a shift from planktonic to sessile state, allowing the formation of more phenotypically complex biofilms.

Keywords: Biofilms, plasmid, Escherichia coli, antibiotic

Acknowledgements: This work has been funded by project PTDC/QUI/69392/2006, from the Portuguese Science and Technology Foundation (FCT).

Pseudomonas fluorescens ER74508 adherence to polymer networks made of polydimethylsiloxane and/or cellulose acetate butyrate

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Pseudomonas fluorescens is a psychrotrophic bacillus frequently found in water and soil, sometimes in association with plants, animals, and man. P. fluorescens isolates from various ecological habitats have the capacity to adhere to and thus contaminate several surfaces, including human respiratory epithelium, plant material, and inert surfaces. The main objective of this work was to study P. fluorescens adherence to polymers as a function of material topography and surface free energy. The previously described P. fluorescens ER74508 clinical isolate was used as the tested strain. Two different chemical polymers were used: n,ω-diacyclat-polydimethylsiloxane (PDMS) and cellulose acetate butyrate (CAB).

The PDMS network was synthesized by free-radical polymerisation with 2,2-Azobisisobutyronitrile (AIBN). The CAB network was synthesized by isocyanate-alcohol addition between the OH function of the CAB oligomers and NCO pluri-isocyanate crosslinker (Desmodur N 3300®). Polymer networks were also synthesized with different mass compositions (w/w): 100% CAB, 75% CAB + 25% PDMS (75/25), 50% CAB + 50% PDMS (50/50), 25% CAB + 75% PDMS (25/75) and 100% PDMS. The unreacted material that remained in the network after polymerization was determined by extraction with dichloromethane. After extraction, the samples were dried under vacuum at 50°C and weighted. The extracted content (EC) is the ratio between the weights of samples before and after extraction expressed as a percentage. EC was lower than 9% for all the networks and than 4% for most of them. All synthesized samples were transparent indicating that there is no phase separation at the microscopic scale.

The physical and chemical properties of the networks were analysed by Dynamic Mechanical Thermal Analysis (DMTA), atomic force microscopy (AFM), and dynamic contact angles measurements. DMTA revealed that the 50/50 and 75/25 networks were correctly interpenetrated, 25/75 samples were inhomogeneous.

These three combinations of polymers have nearly the same surface energy. Among the five studied networks, two distinct profiles of surface topography were observed: the 100% PDMS, 75/25 and 50/50 networks are very smooth with a roughness close to one nanometer while the 100% CAB and 25/75 samples have a roughness of respectively 7 and 20 nm.

Bacterial suspensions calibrated at 10⁸ cells/ml were incubated with the polymer networks at 37°C for 3 hours and adherence was quantified by ATP-metry after elimination of planktonic cells by washings. Two levels of adherence were obtained: maximal adherence to 100% CAB and 25/75 networks and minimal adherence to 75/25, 50/50 and 100% PDMS networks. Adherent bacteria and exopolysaccharides were visualised by epifluorescence microscopy after staining with DAPI, TRITC-conjugated peanut agglutinin and FITC-conjugated Concanavalin A lectins. Bacteria adhered to 100% CAB with a diffuse pattern (adherent bacteria randomly and individually dispersed at the network surface), while bacteria adhered to 100% PDMS, 75/25, 50/50 and 25/75 as microcolonies embedded in exopolysaccharides.

In conclusion, we produced polymer networks with different compositions and determined the relation between the physical and chemical properties of the networks and bacterial adherence. For mixed polymer networks, the nanotopography had a high influence on P. fluorescens adherence: a roughness increase was associated with an adherence increase. An increase of the nanotopography promoted adherence of microcolonies. Smoother surfaces favoured adherence of scattered bacteria. The influence of the nanotopography onto slower colonisation processes i.e. biofilm formation is under study at our laboratory.

Keywords: Pseudomonas fluorescens, polymer networks, adherence, nanotopography.
Quantification of stone biofilms: incomplete factorial designs for the optimization of phytopigment extraction in dimethyl sulfoxide.

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Stone surfaces constitute an adequate environment for the formation of biofilms, which accelerate the deterioration of works of art built on this material. Thus an efficient method for the quantification of photosynthetic growth on rock surfaces is an indispensable tool for the study of the conservation of monuments and the bioreceptivity of building materials. In a previous work we observed that the application of ultrasound improves the extraction efficiency of phytopigments from rock materials, commonly used as biomarkers for the quantification of biomass.

In this work we followed an incomplete factorial design in order to optimize the experimental conditions of phytopigment extraction in dimethyl sulfoxide (DMSO), specifically from rock materials after application of ultrasound. The independent variables were extractant:sample ratio, temperature and duration of extraction. The experimental data allowed the development of empirical models for each phytopigment, describing the interrelationship between operational and experimental variables by equations, including linear, interaction and quadratic terms. The model predicted that the application of ultrasound directly to the intact samples followed by incubation in 0.43 DMSO-sample (vol/surface) at 63°C during 40 min will release at least 90% of total chlorophyll-a. This method will allow to detect and quantify the biocolonization of stone surfaces at early stages.

Quantification of stone biofilms: phytopigments extraction improvement by application of ultrasonic methods.

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Biofilm formation on rock surfaces constitutes a problem for the conservation of monuments and buildings. Chlorophyll-a is a commonly used biomarker for the quantification of photosynthetic growth, however its total extraction from rock materials is incomplete. In this work we assayed the application of ultrasounds in order to improve the extraction efficiency of phytopigments in dimethyl sulfoxide (DMSO).

For this purpose, biofilms were allowed to develop on 6x6x1 cm rock blocks and three mechanical treatments were assayed prior to phytopigment extraction: in the first case, the samples were crushed (control), in the second, ultrasonic bath was applied to the crushed blocks, and in the third treatment, ultrasound was applied directly to the intact block samples with the help of an ultrasonic tip generator. Subsequently, extraction in DMSO was carried out at different experimental conditions and the concentration of chlorophyll-a, chlorophyll-b and total carotenoids was measured with the help of a spectrophotometer. The results achieved allowed to conclude that the application of ultrasounds improves the extraction efficiency of phytopigments from rock materials, even when it is applied directly to the intact rock samples.
Role of planktonic and sessile extracellular signals on interspecies relationships

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It is well established that in nature, bacteria are found primarily as residents of surface-associated communities called biofilms, which are likely to consist of consortia of species that influence each other in synergistic and antagonistic manners. Although few reports specifically address interactions within multispecies biofilms, recent studies have shown that certain bacterial species, as a microbial defence mechanism, secrete extracellular products that interfere with biofilm formation and cell-to-cell communication. This study aimed to evaluate whether synergistic or antagonistic associations occur during multispecies planktonic growth and, single and binary biofilm formation and activity.

P. aeruginosa (ATCC 10145) and Escherichia coli k12 were the strains used in this study. Previously, supernatants obtained under planktonic and biofilm cultures of each single species were recovered, filtered and stored for further experiments. The latter supernatants were supplemented with TSB and used as the growth media to planktonic and sessile growth of both single- and two-species cultures. Planktonic bacterial growth on 96-wells plates was examined through OD600 measurement. Biofilms were obtained after 24 h and evaluated in terms of biomass, through CV and respiratory activity, with XTT. For dual-species growth it was used a combination of 50% of suspended inocula of each species.

Results indicated that both biofilm supernatants had an inhibitory effect on the growth of all planktonic cultures, mainly in the exponential stage. Conversely, single- and two-species planktonic growth has been stimulated in the presence of the P. aeruginosa planktonic supernatant. Concerning biofilm studies, it was found that the supernatants resulting from bacterial biofilm cultures favoured biomass accumulation. This may be due to the release of signalling molecules secreted by bacteria within the biofilms, inducing neighbouring cells to shift from planktonic to sessile growth. None of the supernatants tested had effect in P. aeruginosa biofilms, although all stimulated E. coli biomass accumulation. Concerning E. coli biofilm supernatant, all the biofilms formed in this medium showed biomass increase, more pronounced for dual-species biofilms. Thus, it is possible that E. coli have produced signalling molecules for the bulk media that later may be on the basis of a cooperative biofilm formation by both strains. Regarding respiratory activity, it was observed that this parameter was not significantly altered as biomass values. However, it was noted a slight decrease when the biofilms grew in planktonic supernatants, probably due to the presence of a secondary metabolite released by bacteria in planktonic conditions that may disturb biofilm activity. Since few studies have reported the complex web of interactions within biofilm communities, these results help to understand the behaviour of bacteria when facing microbial defence mechanisms promoted by other species.

Keywords: biofilms; synergistic interactions; biofilm growth


Survival of Listeria monocytogenes in a desserts factory: evaluation of the sanitization treatments employed and of its infrastructure.

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Listeriosis is a rare but clinically important disease caused by Listeria monocytogenes (LM), which produces severe effects on the health of the susceptible population, as immunodepressed people, infants and pregnant women. This bacterium can grow at food-storage temperatures and possess potential for long-term survival, and also has the ability to form biofilms as a protection to disinfectants and antimicrobials. Many foods could be contaminated with LM, but the most dangerous are, principally, the kind “ready-to-eat” (RTE). Epidemiological investigations about food-borne illnesses demonstrated that the presence of vegetative pathogens was frequently due to post-process recontamination, being the surfaces and the environment (probably) the most important elements for these event.

At the present work, we have evaluated a dessert-making factory designed with white rooms, filtered-air positive-pressure (FAPP), hand sanitizer systems, boot washer machines, refrigerated rooms, application of HACCP principles, and different protocols for raw materials treatment. They adopted a program based on two-sort working areas, having different levels of biological risk: the high-risk zones in which happens direct contact between food and surfaces, and low-risk areas within the production facilities (walls, floors, drainage, etc.). The goals were to determine the efficiency of cleaning and disinfection treatments against the persistence of LM in the rooms for food preparation, and to evaluate the pathogen activity and biofilm formation at refrigeration temperature, especially those areas difficult to clean and persistently moist.

Microbiological sampling for LM detection on the surfaces in contact with or without foods, was through smear 10 x 10 cm areas using pre-moistened hyssops, inoculating those into a selective chromogenic broth (Path-Chek Hygiene Listeria Detection Broth), and incubating at 28 - 30º+/-1ºC by 24-48 hours. Positive tests (black colour) were confirmed using API Listeria (ISO 11290.1).

LM was not detected on the surfaces in contact with food, and positive results were restricted to low-risk zones, with 15,2% (N= 164) before the cleaning treatment and 6,9% (N= 333) after doing sanitization. The areas where the bacterium was persistent were circumscribed basically to the floor (either in the sanitary entrance) or in different elements that contacted directly with the floor (shoe soles, steps of stairs, water ponds for shoes sanitizing, beside drains, etc.). There is specially troubled for an integrated hygiene control (it includes baths for shoes sanitizing, automatic hands washing, hands-drying by towel paper and hands sanitizing). All these results show a potential risk to transfer LM from different environments of the factory to the final products.

Keywords: Listeria monocytogenes; survival; food processing; refrigeration temperature
Use of Biosurfactants to reduce adhesion of Staphylococcus aureus to plastic surfaces.

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Introduction. Microbial adhesion to surfaces is considered the main event involved in biofilm establishment. Bacteria growing on biofilms are more tolerant to chemical and physical treatments thus; they are a cause of great concern for food processing and in biomedical fields since bacteria can colonize medical devices and food contact surfaces representing an important source of contamination. Extensive efforts have been directed to avoid bacterial attachment; among them, the change on surface properties by conditioning with biosurfactants (BS) has been studied. Biosurfactants, surface active products of microbial origin, have several advantages over synthetic surfactants. BS have low toxicity, are biodegradable, present chemical diversity, are effective under extreme environmental conditions, such as temperature, pH and ionic strength, show strong surface activity and emulsifying ability and have antimicrobial and anti-adhesive properties. In addition BS can be obtained from renewable substrates, reducing the costs and enlarging the possibility of commercial production. The aim of this work was to verify the potential use of biosurfactants in inhibiting adhesion of growing cells of Staphylococcus aureus ATCC 25923 on polystyrene (PS) surfaces.

Materials and methods. Two biosurfactants were used: Surfactin from Bacillus subtilis and Rhamnolipid (Jeniel BBR599) at 0.1% aqueous solution. Polystyrene microplates were filled with 200µL of each biosurfactant for 24 hours, and after gently washing with water, they were filled with 180µL of TSYE broth. A bacterial suspension was made with water and 20µL of this suspension was inoculated in each well containing the broth and incubated at 35°C for 48 hours. At time defined intervals the wells were washed with water, fixed for 15 minutes with methanol and stained for 15 minutes with violet crystal 1%. After washing with water, the stained wells were discolored with 200µL of acetic acid. The optical density (630 nm) of this stained solution was used for the measurement of adhered cells. Unconditioned plates were used as control.

Results. Results showed that the pretreatment with surfactin solution was able to reduce significantly the adhesion of Staphylococcus aureus to the plastic surface. After two hour contact surfactin reduced the adhesion by 77% however, the reduction level was decreased at long time intervals. The conditioning with commercial rhamnolipid did not show a significant decrease in adhesion levels.

Conclusion. These results suggest that surfactin have good potential as anti-adhesive agent to control Staphylococcus aureus adhesion.

Keywords adhesion; biosurfactants; Staphylococcus aureus; surfactin; rhamnolipids

Acknowledgements to FAPESP for financial support.

Adaptation of Novosphingobium sp. PP1Y to grow on complex mixtures of aromatic compounds dissolved in oil phases

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Aromatic compounds are among the most widespread and dangerous environmental pollutants. Several polymeric aromatic hydrocarbons (PAH), like naphthalene and phenanthrene, are very toxic for aquatic organisms, whereas, benzene, chrysene, benzo[a]anthracene, benzo[a]pyrene and aromatic amines are carcinogenic and represent a direct risk for human health. The main sources of aromatic compounds released into the environment are fuel oil spills which occur not only during accidents involving oil tankers but daily during fuel transfers, thus generating a widespread hydrocarbon pollution.

The so-called "obligate hydrocarbonoclastic bacteria" (OHCB), like Alkanivorax, Marinobacter and Oleispira, are among the most effective oil degraders. However, OHCBs degrade prevalently or exclusively the saturated fraction of petroleum and oil fuels. The most effective degraders of aromatic compounds belong to Sphingomonadales (Gram-), Pseudomonadales (Gram-), and Mycobacteria (Gram+).

As temperature, pH and ionic strength, show strong surface activity and emulsifying ability and have antimicrobial and anti-adhesive properties. In addition BS can be obtained from renewable substrates, reducing the costs and enlarging the possibility of commercial production. The aim of this work was to verify the potential use of biosurfactants in inhibiting adhesion of growing cells of Staphylococcus aureus ATCC 25923 on polystyrene (PS) surfaces.

Introduction. Microbial adhesion to surfaces is considered the main event involved in biofilm establishment. Bacteria growing on biofilms are more tolerant to chemical and physical treatments thus; they are a cause of great concern for food processing and in biomedical fields since bacteria can colonize medical devices and food contact surfaces representing an important source of contamination. Extensive efforts have been directed to avoid bacterial attachment; among them, the change on surface properties by conditioning with biosurfactants (BS) has been studied. Biosurfactants, surface active products of microbial origin, have several advantages over synthetic surfactants. BS have low toxicity, are biodegradable, present chemical diversity, are effective under extreme environmental conditions, such as temperature, pH and ionic strength, show strong surface activity and emulsifying ability and have antimicrobial and anti-adhesive properties. In addition BS can be obtained from renewable substrates, reducing the costs and enlarging the possibility of commercial production. The aim of this work was to verify the potential use of biosurfactants in inhibiting adhesion of growing cells of Staphylococcus aureus ATCC 25923 on polystyrene (PS) surfaces.

Materials and methods. Two biosurfactants were used: Surfactin from Bacillus subtilis and Rhamnolipid (Jeniel BBR599) at 0.1% aqueous solution. Polystyrene microplates were filled with 200µL of each biosurfactant for 24 hours, and after gently washing with water, they were filled with 180µL of TSYE broth. A bacterial suspension was made with water and 20µL of this suspension was inoculated in each well containing the broth and incubated at 35°C for 48 hours. At time defined intervals the wells were washed with water, fixed for 15 minutes with methanol and stained for 15 minutes with violet crystal 1%. After washing with water, the stained wells were discolored with 200µL of acetic acid. The optical density (630 nm) of this stained solution was used for the measurement of adhered cells. Unconditioned plates were used as control.

Results. Results showed that the pretreatment with surfactin solution was able to reduce significantly the adhesion of Staphylococcus aureus to the plastic surface. After two hour contact surfactin reduced the adhesion by 77% however, the reduction level was decreased at long time intervals. The conditioning with commercial rhamnolipid did not show a significant decrease in adhesion levels.

Conclusion. These results suggest that surfactin have good potential as anti-adhesive agent to control Staphylococcus aureus adhesion.

Keywords adhesion; biosurfactants; Staphylococcus aureus; surfactin; rhamnolipids

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Conclusion. These results suggest that surfactin have good potential as anti-adhesive agent to control Staphylococcus aureus adhesion.

Keywords adhesion; biosurfactants; Staphylococcus aureus; surfactin; rhamnolipids

Acknowledgements to FAPESP for financial support.
An integrated approach involving chemical and ecotoxicological evaluation of the efficacy of a bioremediation tool based on bioaugmentation with *Pseudomonas* sp. ADP in soils contaminated with atrazine commercial formulations

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Atrazine, an *s*-triazine herbicide, is among the most frequently detected pesticides in soil and freshwater. Over-use and accidental spills are major threats, resulting in concern regarding the impact of this herbicide and of its toxic metabolites on human and ecosystems health. These facts have promoted search for efficient bioremediation strategies for atrazine polluted environments. *Pseudomonas* sp. ADP is one of the best characterized herbicidal-degrading bacteria. A previous study, based on mineralization assays carried out in small closed microcosms spiked with pure 14C-labelled atrazine, showed that bioaugmentation with *Pseudomonas* sp. ADP combined with soil amendment with organic acids could be a promising approach to cleanup soil polluted with high herbicide concentrations [1]. In the present study, we examined this potential bioremediation strategy in larger open soil microcosms for optimization under more realistic conditions. A representative crop soil from Central Portugal was spiked with two herbicidal commercial formulations, one containing atrazine as single active ingredient [2] and another one also containing the chloroacetoanilide herbicide *s*-metolachlor. Doses higher than the recommended dose for agricultural application, namely between 10- and 200-fold, were tested, mimicking over-use and spill scenarios. Contaminated soils were inoculated with the bacterial inoculum (~1x10^5 CFU/g of soil) and amended with citrate solutions or not amended. To monitor the efficacy of this bioremediation tool, chemical analysis and ecotoxicological assays were carried out in soil samples and in water extracts (elutriates, leachates) from bioremediated and non-soil cleanup based on the ecotoxicological evaluation. Results show that one sole bacterial inoculation or several successive inoculations plus biostimulation, depending on the initial level of soil contamination with the herbicide, allowed rapid (in 1 week) and almost complete removal of atrazine from soil (> 98% of initial), even though atrazine mineralization was apparently not complete in the bioremediated soils [2]. Importantly, in the case of the soils spiked with the mixed commercial formulation, apparently atrazine biodegradation was not accompanied by soil detoxification. Results stress the importance of monitoring ecotoxicity before and after the implementation of a bioremediation approach, besides chemical analysis, to obtain a more realistic glimpse of its potential ecological impact.

Acknowledgments: to FEDER and FCT, Portugal (contracts PPCDT/AMB/56039/2004 and PTDC/AMB/64250/2006 and grants to SC and MMS)

References:

Keywords: atrazine biodegradation, *Pseudomonas* sp. ADP, ecotoxicological assessment, bioremediation efficacy
Applied genetic engineering in the removal of heavy metals

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The heavy metals dissolved in water cause a very aggressive contamination for the aquatic organisms. Some microorganisms present the capacity to accumulate heavy metals in their interior, and later on the heavy metals are passed to more organisms. Therefore, recoveries of heavy metals from wastewater from industries are an important issue for environmental protection and remediation. One of the most popular technique employed for to solve this problem, has been the bioremediation. Biological systems share or arise many peptides and proteins as metal transporters and among those, metallothioneins are one of the most efficient. Metallothioneins are small cysteine-rich proteins with a low molecular weight capable of binding heavy metal ions such as Zn$^{2+}$ and Cd$^{2+}$. They are ubiquitous tissue components in higher organisms, which tentatively have been attributed both unspecific protective functions against toxic metal ions and highly specific roles in fundamental zinc-regulated cellular processes. Metal bind to metallothioneins in a coordinated tetrahedral mode or motif by cisteinic sulfide and such structural coordination allow showing 2 motifs consisting in 11 cystein residues binding to 4 metal ions and 9 cystein residues binding to 3 metal ions. Cellular accumulation process with microorganisms optimized by recombinant technologies could overcome the deficiencies of common metal clean up processes and maybe an alternative for removal and recovery of heavy metals from contaminated water or soil. In this study, genetically engineering Esherichia coli strain was constructed to express a fusion protein consisting of a metallothionein of mouse fused the thioredoxin as the metal-binding-stimulator to create efficient recombinant biosorbents in order to investigate the strains ability to accumulate Cadmium and Lead ions. The recombinant protein was expressed by addition of 1 mM IPTG and the kinetics of sorption was conducted with the recombinant bacteria show that the results of 48% of sorption of cadmium and 99% of sorption by lead. The kinetic of sorption adjust a model of second order that which indicates us a phenomenon of transfer of mass where the likeness of the biomaterial for the metal was $K_{eq}$ = 0.25[L-(mg-h)] as long as $K_{eq}$=0.90[L-(mg-h)]

Keywords Biosorption, Heavy metals, Metallothioneins

Bacterial Reduction of Polycyclic Aromatic Hydrocarbons and Heavy metals in Bonny Light Crude oil Using some Common organic wastes as Bistimulants


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The inevitable exploration and Production of crude oil coupled with daily use of hydrocarbon-based fuels in Nigeria results in serious environmental damage. Thus, oil contamination of agricultural lands and drinking water sources is a common phenomenon particularly in the Niger Delta region. In order to boost agriculture and ensure the provision of potable water, a clean up strategy that employs the use of microorganisms (Bioremediation) appears to be more promising. One of the ways by which Bioremediation can be enhanced is through addition of nutrients. This is why some selected organic wastes that are nutrient rich were used in this work with a view to assessing their effect on PAHs and heavy metals reduction. Samples of brewery effluents, cow dung, sewage sludge and chicken droppings were obtained and dried. Each sample was ground into powder and kept in polythene bags. Different concentrations of the samples (3.22gL$^{-1}$, 6.44gL$^{-1}$, and 12.88gL$^{-1}$) were prepared and used as a basal degradation medium (BDM). About 10ml of this medium was dispensed in universal bottles and 0.5ml of Bonny light crude oil was then overlaid in each bottle and seeded with crude oil degrading bacteria. After seven days the residual crude was extracted and subjected to chromatographic analysis using GC-MS methods. A significant (P<0.05) reduction in Polycyclic Aromatic Hydrocarbons (PAHs) and heavy metals was achieved. The results obtained indicate that these organic wastes have a potential as Bioenhancement agents in Bioremediation.

Keywords Polycyclic Aromatic Hydrocarbons, Bioremediation, Organic wastes
Bias in analytical procedure of microorganism's community structure in soil

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When the bioremediation is done, it is necessary to evaluate the influence that the clean-up work gives to human health and to evaluate the ecosystem effect. In this work, we examined the method for analyzing to understand the influence that the bioremediation gave to an environmental (soil) microorganism group accurately in this announcement. The method for analyzing consists of the following four processes, collection of environmental (soil) sample, DNA extraction from the sample, DNA amplification by PCR and statistics processing of electrophoresis Profile by denaturing gradient gel electrophoresis (DGGE) or terminal restriction fragment length polymorphism (T-RFLP). In this announcement, DGGE was adopted and examined that bias in the above-mentioned of four processes and influence to the microorganism group structure analysis.

In the collection of the soil sample, we examined the influence of soil granules and sample source on the bacterial and fungal floras. Pulverizing granules by hand and deference of sample collection position altered the fungal flora, but had no effect on the bacterial flora. In the process of DNA extraction from the sample, when we compare the microorganism group structures by using DNA extracted from a kit on the market, as a result, the difference was admitted in the soil that did not do DNA extraction easily though the difference was not admitted in the microorganism group structure between the kits for the soil that did DNA extraction easily: Moreover, the addition of skimmed milk has improved extraction efficiency of DNA to the soil with a difficult DNA extraction. In the process of DNA amplification by PCR, we compared DGGE fingerprints by 18S rDNA and internal transcribed spacer (ITS) region from same extracted DNA sample. Both of fingerprints moved statistical analysis. In the process of statistics processing of the DGGE profile, principal components analysis (PCA) and multidimensional scaling (MDS) gave different statistical results.

Keywords DNA extraction; PCR-DGGE; statistical analysis; bioremediation; microorganism structure
Biodegradation of Polyvinyl chloride (PVC) by newly isolated fungal strain of Lentinus tigrinus PV2

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Polyvinyl chloride (PVC) is one of the most important synthetic plastics causing environmental pollution when they are disposed off as waste. The present study was conducted to study the biodegradability of the newly isolated fungal strain for PVC. Fungus was isolated from plastic waste contaminated soil. The strain was identified on the basis of morphological and molecular characterization by rRNA as Lentinus tigrinus PV2. The biodegradability of the strains was checked in shake flask experiments with Mineral Salt Media (MSM) with PVC as a carbon source. The biodegradation was analyzed by surface changes (Visual and scanning electron microscopy), utilization of PVC as carbon source (biomass quantification and starch test). Further more the structural and molecular weight changes in PVC film were analyzed by Fourier transform infrared spectroscopy (FTIR) and gel permeation chromatography (GPC). The results showed that Lentinus tigrinus PV2 has good degradation potential of PVC and could be used as candidates for bioremediation of plastics waste.

Keywords: Biodegradation, Polyvinyl chloride, Fungus

Biodegradation of two herbicides: Metribuzin and Linuron by some Fungal Species isolated by a Polluted soil in the North - East Algerian

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The use of micromycetes with the aim of the biotransformation of pollutants of the environment is a domain in full expansion. Numerous studies were realized on stumps degrading xenobiotic such as the aromatic compounds, the by-products of the lignine and the hydrocarbon aromatic polycyclic. For our part, we were interested to other toxins such as triazines and phenylureas. To do it, the bioremediation of these two herbicides from liquid media by soil fungi belonging to various taxonomic groups was determined.

The evaluation of the disappearance of the herbicides was realized by liquid chromatography high-performance (HPLC). The obtained results reveal that the metribuzin is degraded by the majority of stumps tested with rates not exceeding the 38 % with the exception of Botrytis cinerea (61 %). As regards the linuron, four stumps are able to convert it to more than 25 % whereas Botrytis cinerea transforms it almost completely (91 %). The majority of the profiles HPLC revealed the appearance of new peaks during the disappearance of compounds, so indicating their transformation by fungal species. However, from our results, it is not excluded that another process as that of the bioaccumulation is implied in the processes of disappearance of compounds.

Keywords: pollution, Soil, Herbicides, Metribuzin, Linuron, Biodegradation, Fungi
Bioremediation of heavy metals through symbiosis between leguminous plant and rhizobium with engineered metallothionein and phytochelatin synthase genes

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Toxic metal contamination in agricultural fields is an important worldwide problem. Particularly, cadmium contamination in rice grains is the most important issue in Asian countries. We developed a novel bioremediation system, “symbiotic engineering”, based on the symbiosis between leguminous plant, Astragalus sinicus and the recombinant rhizobium, Mesorhizobium huakuii subsp. rengei B3, by overexpressing a synthetic tetrameric metallothionein gene (MTL4) and cDNA encoding the phytochelatin synthase from Arabidopsis thaliana (AtPCS). The MTL4 and AtPCS genes were fused to the nodule-specific expressing promoter, nolB or nolH promoter. By the expression of the MTL4 and AtPCS genes, MTL4 protein and phytochelatin ([γ-Glu-Cys]x, γ - PCs) were formed in the recombinant rhizobium and increased the ability of cells to bind Cd2+ by 9- to 19-fold approximately. When the recombinant rhizobium established the symbiotic relationship with A. sinicus, the symbionts increased Cd2+ accumulation in root nodules by 1.5- to 2 fold. The expression of the both MTL4 and AtPCS genes showed additive effect on Cd2+ accumulation in nodules. Introduction of the iron-regulated transporter gene from A. thaliana (AtIRT1) in the recombinant rhizobium advanced the accumulation of Cu2+ and As3+ in the nodules of A. sinicus. In rice paddy soil, addition of recombinant strain B3 carrying a plasmid with the both MTL4 and AtPCS genes significantly increased the accumulation of cadmium in roots and nodules of A. sinicus. Thus, this system uses the advantages of both plants and rhizobium. In particular, the system can transform engineered genes to the host plant through infection with a recombinant rhizobium.

Bioremediation of Polyethylene

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Polyethylene is one of the most consuming plastics due to its durability and excellent mechanical properties together with price-competitiveness and good processability. It is being widely used for many single use receptacles, packagings, fishing tools and agricultural films. However due to the recently grown up environmental pressure, eventual fate of plastics after use became more and more important. It has been admitted that the molecular weight of polyethylene should be lower than 500, otherwise it should be at least formulated with transition metal pro-oxidants and then oxidized to low molecular weight so as to be biodegraded. A lot of researches have been carried out to examine biodegradation of polyethylene containing pro-oxidants and to explore microorganisms degrading polyethylene having deliberately been oxidized.

In this study, a thermophilic bacterium able to degrade low-molecular-weight PE(LMWPE) was isolated from compost. LMWPEs were prepared by thermal degradation of HDPE and LDPE under strict nitrogen atmosphere. Biodegradation of LMWPEs whose average molecular weights were well above 500 was examined by measuring the amount of CO2 evolved from compost loaded with LMWPEs after inoculation with the isolated strain. A microorganism isolated from the compost made from animal fodder formed clear zone with about 4 mm of diameter at 58°C. The isolated strain was a rod shaped Gram negative bacterium. Based on the results of the 16S rDNA analysis, the isolated strain was identified to be Thermophilic bacterium Methylobacterium chloromethanicum. It is an aerobic bacterium and is known to consume methanol and methylamine together with other C2, C3, C4 compounds for growth.

The bacterium acclimated with a low molecular weight PE1 was active not only for the degradation of PE1 but also for the other higher molecular weight LMWPEs, PE2, PE3 and PE4. However the biodegradation decreased with increase in the melt viscosity of the LMWPEs. The low molecular weight fraction of PE1 decreased significantly as a result of the degradation, and the molecular weight distribution of PE1 after the degradation was much narrower than that of PE1 before the degradation. The weight-average-molecular weight (Mw) of PE1 increased from 1,700 to 2,400 during the biodegradation. This should be attributed to the preferential assimilation of the low molecular weight fraction of PE1 by the microorganism.

FTIR spectra of LMWPE before and after the biodegradation were normalized with respect to the peaks at 2830 and 2920 cm⁻¹ corresponding to C-H stretching. A LMWPE exhibited small peaks at 719 and 729 cm⁻¹ corresponding to alkynes disclosing that some hydrogen atoms were dehydrogenated forming unsaturated bonds during the thermal degradation of PE to prepare LMWPE. After the biodegradation of LMWPE, new peaks appeared on the FTIR spectra at 1095 and 1026 cm⁻¹ assignable to C-O stretching revealing that some carbons in LMWPE were oxidized by the microbial action of the isolated strain.

Keywords Polyethylene; Biodegradation; Methylobacterium chloromethanicum; Thermophilic bacterium
Biosorption of mercury by bacteria and potential applications for bioremediation

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Heavy metals, and particularly metallic mercury and its organic derivatives, are responsible for acute toxicity and heavy metal pollution represents a public health and wildlife concern. Bacteria resistant to heavy metals constitute an attractive approach to develop systems to decontaminate soils, sediments or water [1]. Biosorption of heavy metals by bacteria or bacterial biomass have been developed for the bioremediation of contaminated water [2,3].

Here we selected and characterized bacteria displaying mercury biosorption propensity. The isolation of bacteria from soil, effluents and river sediments contaminated with heavy metals (Rio Tinto mining area, Spain) permitted to select bacteria for their resistance to mercury (MICs in the 20 – 100 µM range). Seven strains were further selected for their mucoid phenotype indicative of biosorption propensity. The bacteria were identified by 16s rDNA sequencing. The fixation of mercury was quantified by inductively coupled plasma-optical emission spectroscopy and the mercury extracellular sequestration was characterized by transmission electron microscopy in conjunction with X-ray energy dispersive spectroscopy. Bacteria grown in the presence of mercury generated mercury extracellular sequestration as HgS precipitates and/or beads. Non-living bacterial biomass incubated in the presence of mercury only generated extracellular sequestration of mercury beads, with a biosorption yield superior to that of the living bacteria (40-120 mg Hg per g of dry biomass). These results permit to propose biosorption methods to remove mercury from contaminated water.

Keywords: bioremediation; decontamination; mercury; toxic metals; resistant bacteria.

References

Biosurfactant production by Pseudomonas fluorescens – Physico-chemical characterization and solubilization of a model organic compound, naphthalene

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Biosurfactants are amphiphilic compounds with surface activity; they are produced by a large number of microorganisms, including bacteria, yeasts and fungi. Biosurfactants production is an important area of research, owing to the large number of potential applications, especially as substitutes for synthetic surfactants in oil and other industries. Pseudomonas are the best-known bacteria capable of utilizing hydrocarbons as carbon and energy sources and producing biosurfactants to enhance the uptake of such immiscible hydrophobic compounds.

Production of biosurfactant by free and alginate entrapped cells of Pseudomonas fluorescens Migula 1895-DSMZ was investigated using olive oil as a sole carbon and energy source. Biosurfactant synthesis was followed by measuring surface tension and emulsifying index E24 over five days at ambient temperature and neutral pH. The properties of biosurfactant that was separated by acetone precipitation showed that the biosurfactant was a glycolipopeptid-type, and had a good foaming and emulsifying activities. The critical micellar concentration (CMC) was found 290 mg L-1. The biosurfactant also showed good stability during exposure to high temperatures (up to 120°C for 15 minutes), high salinity (20% NaCl) and a wide range of pH (4-9).

Wetability and solubility tests were also conducted and showed that the isolated biosurfactant decreased the surface hydrophobicity of polystyrene and increased the solubility of a model organic compound, naphthalene, in aqueous solutions. Above the CMC, naphthalene solubility was deeply affected by biosurfactant concentration (3 to 7 times its aqueous solubility), pH and salinity. The solubility reached a saturation value (205 mg L-1) when biosurfactant concentration exceeded 1.5 g L-1. For alkaline pH or high salinity (above 10%) the solubility decreased by more than 50%. The weight solubilization ratio decreased from 0.63 to 0.015 for increasing biosurfactant concentration up to 1.5 g L-1, alkaline pH or high salinity; and reached a constant value for 4.0 g L-1 biosurfactant irrespective of pH and salinity. In all cases, the solubility of naphthalene in water was enhanced by the biosurfactant addition, showing its potential for application in bioremediation of polycyclic aromatic hydrocarbons contamination in extreme environments.

Keywords: Biosurfactant; Pseudomonas; Physico-chemical characterization; Naphthalene; Stability; Solubility.
Changes in microbial populations over time in an AMD affected field site

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Microbial communities play an essential role in the global metabolic cycles of carbon, nitrogen, sulfur and other elements. Especially in contaminated regions, microbes are important parts of the ecosystem influencing element mobility. The microbial communities have to cope with these contaminants, which are providing an additional selective pressure.

At our study and sampling site near Ronneburg (Thuringia, Germany), heavy metals were mobilized via leaching processes driven by acidic mine drainage (AMD) and microbial leaching with Acidithiobacillus ferrooxidans. During the leaching process, the leachate percolated through the isolation layer and contaminated the underground with mobilized heavy metals and salts.

After removal of the heap material, the salt and heavy metal rich sediment led to a pH in the range of 3 to 4.5, while the content of organic matter is very limited. As a result of these parameters an obvious decrease in numbers of cfu per gram soil of 100 to 1000 fold were observed in comparison to an uncontaminated soil. The test field Gessenwiese was installed 2001. Three large plots were prepared with different treatments: 5cm with topsoil or compost and no amendment as control.

To understand the interdependencies between affecting conditions and to investigate the influence of heavy metals, the population dynamics and growth characteristics of single isolates were investigated including both cultivation-dependent and DNA-based fingerprinting methods. Plating strain isolation, direct cell counts and respiration measurements were used to establish surface and vertical profiles at the heavy metal contaminated field site to follow microbial diversity over time.

Long time observation of the living cells showed that the cell number decreases slowly after addition of compost. The positive effect of the compost and topsoil addition to fertilize substrates and promote plant growth can still be visualized of plant growth. Plating experiments showed a majority of Gram-positive bacteria with the ability to form spores. With molecular methods, it was able to detect also Gram-negative microbes, which usually are predominant and widespread in soil. Cloning of 16S rRNA genes helps to describe the entire microbial community. A 16S rRNA gene bank is set up to identify the active microbial consortia.

Keywords: acid mine drainage, heavy metal, 16S rDNA

Characterization of Ni-resistant plant growth promoting bacterium Bacillus megaterium for microbial-assisted phytoremediation of Ni contaminated soils

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The use of microorganisms for the remediation of metal contaminated soil is an area of extensive research and development. A nickel-resistant bacterial strain SR28C was isolated from a nickel rich serpentine soil in the northeast region of Portugal. Based on the morphological, biochemical characteristics and comparative analysis of partial 16s rDNA sequence with already available database, the microorganism was closely related to Bacillus megaterium. The strain B. megaterium SR28C tolerated concentrations up to 1200 mg Ni L\(^{-1}\) on a Luria-Bartani (LB) agar medium and biosorbed a substantial amount of nickel. Besides, B. megaterium SR28C showed high degree of resistance to various metals (Cu, Zn, Cd, Pb and Cr) and antibiotics (ampicillin, tetracycline, streptomycin, chloramphenicol, penicillin and kanamycin) tested. In addition, the strain exhibited the solubilization of phosphorus and production of IAA in NBRIP medium and LB medium, respectively in the absence and presence of nickel. Concurrent production of IAA and the solubilization of phosphorus revealed its plant growth promotion potential. In pot experiment, inoculation of plants with B. megaterium SR28C significantly promoted the growth of Brassica juncea, Luffa cylindrica and Sorghum halepense in Ni contaminated soils. However, the maximum increase in shoot and root biomass was observed in L. cylindrica. In addition to plant growth promotion, the strain SR28C protected the test plants from Ni toxicity by reducing the uptake of Ni by plants. Thus, the innate capability of this novel isolate for parallel bioremediation and plant growth promotion has significance in the management of environmental and agricultural problems.

Keywords: Nickel; Antibiotic resistance; Bacillus megaterium; Phosphate solubilization
Chlorpyrifos degradation in a biomix of biobed system with allophonic top soil
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Studies in several parts of the world, mainly in Sweden, have demonstrated that biobeds can effectively treat pesticide waste arising from accidental spillages. The biobed is a simple biological system composed by biomixture of top soil, straw and peat, providing a matrix to adsorb the pesticides and facilitate their biodegradation. The straw stimulates the growth of lignin-degrading fungi and the formation of the ligninolytic enzymes; the soil provides sorption capacity and should have a humus and clay content that promote microbial activity, and is also an important source of pesticides degrading microorganisms, that can act synergistically with the fungi; the peat contributes to sorption capacity, moisture control and also abiotic degradation of pesticides. Therefore, the aim of this study was performed to determine whether biomix of biobeds system with allophonic top soil (Andisol) can degrade effectively the organophosphorous insecticide chlorpyrifos.

The biomix was conformed of straw (50% v/v), peat (25% v/v) and topsoil (25% v/v). The biomix was pre-incubated for 0, 15 and 30 day at 20 ± 1 °C before to be contaminated with chlorpyrifos (160 mg kg⁻¹) and the chlorpyrifos degradation was evaluated during 30 days. Besides, the degradation of chlorpyrifos in biobed system at the concentration of 320 and 480 mg kg⁻¹ was evaluated. CO₂ evolution, enzymatic activities (manganese peroxidase (MnP) and fluorescein diasetate hydrolysis-FDA), residual concentration of chlorpyrifos and TCP (3,5,6-trichloro-2-pyridinol) formation were periodically evaluated in both studied.

Degradation of chlorpyrifos (160 mg kg⁻¹) was quicker with 15 day of pre-incubation of the biomix (90%). Instead, the degradation with 0 and 30 days of pre-incubation were 83 and 80%, respectively. The maximum of TCP formation occurs after 10 days of incubation, in the tree pre-incubation period evaluated, with values of 49, 27 and 47%... time, degradation of TCP was observed in all assays. MnP activity was highest in 0 day of pre-incubation period (11 U Kg⁻¹ of soil), instead at 15 and 30 days the activities were 8 and 3 U Kg⁻¹ of soil, respectively. Initial FDA activity was 9.5, 6.3 and 2.5μg g⁻¹ h⁻¹ at 0, 30 and 15 days of pre-incubation period, respectively. No significant differences were observed in C0₂ formation.

The degradation of chlorpyrifos 320 and 480 mg kg⁻¹ occurred efficiently in biobed system (> 90%) after 40 days of incubation period. In both assays TCP formation was detected, however, over 10 days degradation of TCP was observed in both assays. Significant differences were not found in CO₂, MnP and FDA activities, but them increased in the time.

In conclusion, the results of this study demonstrate that pre-incubation time of biomix with allophonic soil between 0-30 days had no major effect on the chlorpyrifos degradation. High concentration of chlorpyrifos can be degraded in biobed. TCP was formed in the biobed system, but it also can be also degraded by this system.

Keywords: Biobeds; Allophonic soil; chlorpyrifos.

Acknowledgements: Investigation financed by Fondecyt 1090678, Fondecyt 3090049.
Complete Dechlorination of Tetrachloroethene and Trichloroethene by Korean Dehalococcoides spp. in a Chloroethene-contaminated Freshwater Sediment

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Tetrachloroethene (PCE) and Trichloroethene (TCE) are one of the most common contaminants in the environments. Contamination of groundwater aquifers by PCE and TCE is a serious environmental concern. It is known that chlorinated ethenes can be reductively dechlorinated to less chlorinated compounds including ethene, which is a nontoxic end-product. Under anaerobic condition, microorganisms use chlorinated ethenes as the terminal electron acceptors and then energy is produced for the cell growth. This process is known as chlororespiration. During the process, the accumulations of intermediates, such as cis-1,2-Dichloroethene (cis-DCE) and Vinyl Chloride (VC), should be avoided, since those intermediates are recalcitrant and carcinogenic. Interestingly, it is known that no microorganisms but Dehalococcoides spp. can dechlorinate beyond cis-DCE. Nevertheless, no Dehalococcoides isolates have been obtained outside North America and little is known about the presence, the distribution, and the dechlorinating activities of Dehalococcoides spp. in other continents. In this study, we attempted to obtain the enrichment cultures that completely dechlorinate PCE and TCE to ethene and to compare Dehalococcoides populations in Korea with previously characterized Dehalococcoides spp. The freshwater sediments were collected from Wonju Streams, located by Woosan Industrial Complex. Approximately 10 g of sediment was used to establish anaerobic microcosms. All microcosms filled with sodium bicarbonate buffer (5mM, pH 7.2, anoxic) up to 100 ml. A set of microcosms were amended with 0.13 mM of PCE and a second set of microcosms received 0.18 mM of TCE. Heat-treated microcosms served as negative controls. Microcosms were incubated at room temperature for 50 days. In every one of two weeks, 0.1 ml of Headspace gas was taken to analyze the dechlorinating activities using a gas chromatography. After the dechlorination was observed, Korean Dehalococcoides populations, involved in the dechlorination process, were characterized based upon 16S rRNA genes. For identification and quantification, the microbial community structure analysis, nested PCR, and quantitative real-time PCR were conducted. During the incubation period, PCE and TCE were quickly dechlorinated to ethene. The microbial community shifts and nested PCR result show that Korean Dehalococcoides populations were responsible for the complete dechlorination. These populations seemed quite similar to the previously characterized Dehalococcoides spp. in 16S rRNA sequences. The qPCR results also support that Dehalococcoides populations were grown during the incubation period in respond to PCE or TCE addition. These results imply that Dehalococcoides spp., which is responsible for the complete dechlorination, exist in not only in North America but also other continents and that the presence of Dehalococcoides spp. is essential for the complete detoxification of chlorinated ethenes.

Keywords Tetrachloroethene; Trichloroethene, Reductive Dechlorination, Dehalococcoides spp.

Degradation of fluoroanilines by the wild strain Labrys portucalensis

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Aromatic amine compounds, many of them with halogenated substituents, constitute a major class of environmental pollutants that have been released into soil and water due to extensive use in industries and agriculture. Biodegradation has been found to be a major route for the removal of this kind of toxic and recalcitrant pollutants from the environment.

Whereas the degradation of mono- and dichlorinated anilines has been studied, very little is known about fluorinated anilines. Therefore, the objective of this study was to investigate, under aerobic conditions, the degradation of 2-, 3- and 4-fluoroaniline by a previously isolated pure bacterium, designated as strain F11. This microorganism, identified as Labrys portucalensis, was isolated from a contaminated site in northern Portugal and has the unique capacity to utilize fluoro benzene as a sole carbon and energy source. The results of the biodegradation of 2-, 3- and 4-fluoroaniline by strain F11 showed that this microorganism is able to completely degrade 2- fluoroaniline and partially degrade 4-fluoroaniline, when these compounds are present as a sole carbon and energy source. Biodegradation of these two compounds also occurred, although at a lower rate, in the absence of an external nitrogen source in the culture medium.

To our knowledge, this is the first study reporting the biodegradation of 2- and 4-fluoroaniline as a sole carbon and energy source by a pure microbial culture.

C.L. Amorim and M.F. Carvalho wish to acknowledge a research grant from Fundação para a Ciência e Tecnologia (FCT), Portugal (Ref. SFRH/BPD/44670/2008, respectively) and Fundo Social Europeu (III Quadro Comunitário de Apoio). This work was supported by the FCT Project - PTDC/BDI/67836/2006

Keywords: biodegradation; fluorinated anilines; environmental pollutants.
Degradation of hydroquinone in *Sphingomonas* sp. strain TTNP3

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A number of pollutants of environmental concern, e.g. *p*-nitrophenol, pentachlorophenol, hexachlorocyclohexane, certain nonylphenols, and bisphenol A, are microbially degraded via hydroquinone (HQ) as a key metabolite. Oxidative degradation of HQ proceeds either via hydroxylation of HQ and subsequent ring opening or via direct ring cleavage without prior hydroxylation. In contrast to the extensive information on bacterial degradation of catechol and its derivatives however, current knowledge on hydroquinone degrading enzymes is scarce and only few relevant enzymes have been characterized.

The activated sludge isolate *Sphingomonas* sp. strain TTNP3 uses a type II ipso-substitution mechanism to degrade the endocrine disruptors nonylphenol and bisphenol A to HQ, which serves as a carbon and energy source. Here, we present the metabolites that are formed during HQ degradation by TTNP3 and discuss specific characteristics of the ring-cleaving hydroquinone dioxygenase.

Both raw and partially purified cell extracts of *Sphingomonas* sp. strain TTNP3 showed HQ degradation activity. We analyzed the corresponding metabolites and identified them using HPLC-radiodetection and GC-MS. Whereas raw cell extract transformed HQ to 3-oxoadipate via 4-hydroxymuconic acid semialdehyde and maleylacetate, partially purified cell extract yielded only 4-hydroxymuconic acid semialdehyde. This result indicates that HQ is subjected to ring-cleavage by a hydroquinone-1,2-dioxygenase in strain TTNP3.

**Keywords** endocrine disruptors, degradation, hydroquinone, dioxygenase

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Degradation of vapour phase toluene in a sustainable organic biofilter media

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Degradation of vapour phase toluene was studied in a sustainable organic biofilter media under continuous and pulse feed mode. Coir pith natural micro-flora (2.3×10⁶ CFU), high water holding capacity (700-750% w/w), moisture content (83%), porosity (80 %) etc. were ideal for application in biofilter. The biofilter does not require any addition of nutrients except the bio-sludge loaded at the start up stage. 100% toluene removal was achieved up to a filter volumetric loading of 103.5 g toluene/m³.h. The CO₂ produced accounted 60% (w/w) of the theoretical CO₂ from corresponding toluene (assuming complete mineralization of toluene). At a tested maximum of toluene loading of 120.72 g/m³.h, 80% removal was achieved. Furthermore, inlet toluene conc. ranging from 0.725-2.63 g/m³ was removed in the biofilter without any toxicity effect in short acclimation period (4-7 days). Biofilter media with coir pith and activated carbon performed better than media with pith alone. Toluene loading under different 1 hr. pulse feeding cycles (15, 5 and 1 minute on time) indicated the biofilter can handle shock loading conditions. However, more retention time is required for complete degradation under higher loading conditions. During continuous operation of the biofilter, the pH of the medium was around neutral range and the pressure drop was negligible (0-4 mm H₂O). The toluene degrading microbial community consisted of 4 *Pseudomonas* sp. The grazing fauna in the coir pith based biofilter media composed of ciliated protozoa (*Colpoda inflate*, *Euplotes harpa* and *Acineria sp.*). The micro-metazoan community was represented by *Nematodes*, *Rotifers*, *Tardigrades* and *Fly larva*. The dynamic grazing fauna exhibited a unique spatio-temporal distribution throughout the biofilter column. The grazing fauna is considered to have enabled the smooth start up of the coir pith based biofilter media by providing nutrient recycling. The present study reveals coir pith medium is an excellent renewable alternative for peat as biofilter media. Also, inherent problems of biofilters during continuous operation such as filter bed clogging and nutrient limitations could be addressed with sustainable grazing community in the filter bed.

**Keywords** biofiltration, Toluene, grazing fauna
Diversity of methylotrophic bacteria isolated from mangrove species and their potential for bioremediation of heavy metals

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The high biological productivity of the mangrove ecosystem demands a high nutrient availability at the beginning of the trophic chain. Microorganism activities are essential in the organic matter decomposition process and nutrient fixation. Bacteria species adapted to mangrove conditions present a potential source of new biotechnologies resources, such as bacteria species that produce valuable enzymes. The genus *Methylobacterium* comprises the PPFMs (pink-pigmented facultative methylotrophic) bacteria. These bacteria can fix nitrogen, nodulate the host plant, produce cytoxinin (a plant hormone) and enzymes such as pectinase and cellulase, as well as be used on the bioremediation of polluted environments due to its metal tolerance. These characteristics make *Methylobacterium* spp. important for plant growth promotion. To further examine these characteristics, *Methylobacterium* spp. were isolated from the mangrove species (Figure 1) such as *Rhizophora mangle*, *Laguncularia racemosa* and *Avicenia* sp. as endophytic bacteria. Samples of mangrove species were collected in Bertioga, São Paulo, Brazil, from locations either contaminated and uncontaminated by oil spills. The tolerances of the isolates to different heavy metals were assessed by exposing them to cadmium (Cd), lead (Pb) and arsenic (As) in different concentrations (0,1mM, 0,3mM, 1mM, 2mM, 4mM and 8mM) (Figure 2). Additionally, the genetic diversity of *Methylobacterium* spp. by the sequence analysis of the gene 16S rRNA (ribosomal RNA gene) was analyzed. The isolates from the oil spill locations were grouped, suggesting that oil can select microorganisms that tolerate oil compounds and change the methylotrophic bacteria community. Cadmium is the most toxic heavy metal assessed in this work followed by arsenic and lead, and two isolates are tolerant to cadmium, lead and arsenic concentration assessed (Figure 3). These isolates have the potential to be used for bioremediation of mangrove environments that are contaminated by oil spills by immobilizing heavy metals present in the oil.

**Keywords:** bacteria diversity, 16S rRNA, *Methylobacterium*, endophytes, plant-bacteria interaction

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Dynamic model of multi-trophic interactions in bioremediation food webs

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Non-steady state mathematical models were developed to examine the dynamics of organic pollutant utilization, microbial competition, inhibition and predation in a multi-trophic system populated by bacteria of different growth rates and ciliated protozoa in continuously mixed flow and batch reactors under different conditions. Assuming that nitrogen and phosphorous were present in excess, the biodegradation of naphthalene was examined as a model organic pollutant (substrate). As slow growing bacterium can co-exist in the system along with a fast growing bacterium in the presence of ciliate predators only if they differ in one of several ways. Inhibition of the fast growing prey, selective predation on one of the prey species by the ciliate and predation defense mechanisms adopted by the bacteria can allow co-existence of the two prey species in the system along with the ciliate predators. Interestingly, co-existence results in enhanced naphthalene degradation. In natural systems, prey species under predation pressure are typically thought to co-exist only due to differences in resource use. It is not only the partitioning of resource use, or predation alone that allow the co-existence of two prey species together; but rather both processes operating simultaneously. These models predict that grazing of bacteria by protozoa can enhance the process of bioremediation similar to the results of some experimental studies, and predation plays an important role in determining the bacterial community structure in such systems. Further, this supports the idea that increased species diversity may increase the ability of microbial ecosystems to degrade pollutants.

**Keywords** microbial food web; bioremediation; predation; protozoa; biodegradation; naphthalene
Enhanced Diodegradation of 1,2-Dichloroethane in Soil Contaminated with Heavy Metals under Different Bioremediation Strategies

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1,2-Dichloroethane (1,2-DCA) represents one of the world’s most important toxic pollutants and is produced annually in excess of 5.443×10^9 kg per year, quantities larger than that of any other chlorinated aliphatic compound. The major concern over soil contamination with 1,2-DCA stems largely from health risks as it has been identified as a potential carcinogen to humans. Owing to their toxicity, persistence and potential for bioaccumulation, there is a growing interest in technologies for their removal. Co-contaminated environments are considered difficult to remediate because of the mixed nature of the contaminants and the fact that the two components often must be treated differently. Therefore, the focus of this study was to assess the impacts of arsenic and cadmium on the aerobic biodegradation of 1,2-DCA, by indigenous microorganisms, in co-contaminated soil undergoing different bioremediation treatments. 1,2-Dichloroethane was degraded readily in both As^(3+):Cd^(2+) contaminated loam soil with the degradation rate constants ranging between 0.0813 - 0.204 day^-1 and 0.091 - 0.216 day^-1, respectively. The presence of heavy metals have a negative impact on 1,2-DCA degradation, resulting in up to 9.31% reduction in 1,2-DCA degradation, after 20 days. Biostimulation and treatment additives increased 1,2-DCA degradation, with the best degradation observed upon the addition fertilizer and calcium carbonate, resulting in up to 14.92% and 16.95% increase in 1,2-DCA degradation, respectively. Bioaugmentation proved to be the better option with dual-bioaugmentation being most effective resulting in up to 37.50% increase in 1,2-DCA degradation, after only 5 days. An initial decrease in urease activity was observed from day 0 to day 5, followed by a steady increase for all treatments except for soils biostimulated with fertilizer. Dual-bioaugmented soils caused a 22.95% and 8.48% increase in total 1,2-DCA degrading population in As^(3+):Cd^(2+) co-contaminated soil, respectively. Results from this study have promising potential for effective remediation of soils co-contaminated with chlorinated organics and heavy metals. However, the best bioremediation strategy will depend on the soil types, microbial population present in the soil matrices, nutrient availability and metal forms.

Evaluation of toxic compounds effects on aerobic granule activity

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Aerobic granule formation is used as a novel technology for the biological treatment of wastewaters. Aerobic granular sludge presents several advantages towards activated sludge, such as excellent settling properties, high biomass retention, ability to deal with high organic loading rates and to perform simultaneously diverse biological processes, such as COD, N and P removal. Researchers have focused mainly in the biological processes in domestic wastewater treatment. However, these studies are not directly applicable to industrial wastewaters due to their specific composition. Several industries are dealing with a high concentration of salt, pH and other toxic compounds, like fluorophenols, in their wastewater. Xenobiotics can inhibit the biological processes of the plants treating these industrial wastewaters. Preliminary studies reported in the literature have shown that granules seem to be more resistant to toxic effects of phenol than flocculated sludge, mainly because of the compact and shielding structure of the granules.

In this work, the short term effects of salt, pH and 2-fluorophenol (2FP) on the conversion processes of nitrification and phosphate removal by aerobic granules were investigated and compared to activated sludge systems. Experiments were conducted in batch mode with granular sludge and crushed granules, obtained from a pilot plant in The Netherlands, and with activated sludge, obtained from a wastewater treatment plant (WWTP) also located in The Netherlands. Ammonium consumption, nitrate and nitrate production and phosphate removal were measured along the 2 h experiments. Overall, the results showed that granular sludge is less affected by toxic compounds than activated sludge. Measurements of the oxygen uptake rate (OUR) inside the granules and studies on the change of granules morphology are ongoing.

Keywords: Granular sludge; Activated Sludge; Toxicity; 2-fluorophenol; pH; Salinity

Acknowledgements:
A.F. Duque and M.F. Carvalho wish to acknowledge a research grant from Fundação para a Ciência e Tecnologia (FCT), Portugal (Ref. SFRH/BD/30771/2006 and SFRH/BPD/44670/2008, respectively) and Fundo Social Europeu (III Quadro Comunitário de Apoio). This work was supported by the EU project INNOWATECH – Contract-No. 036882.
Extracellular production of hydroxyl radical by Streptomyces cyaneus CECT 3335 via quinone redox cycling: a new strategy for BTEx remediation

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The growing demand of society for the design of strategies for degradation of persistent organic pollutants detected in waters and/or soils is reflected in stringent governmental regulations. The strategies to solve this problem is addressed to development of new degradation technologies based on oxidative (physical-chemical and biological processes, including the so-called "Advanced Oxidation Processes" (AOP) based on the production of oxygen reactive radicals. Among biological approaches, procedures which involve the oxidative potential of ligninolytic microorganisms are being explored [1, 2, 3]. The degradation capability of ligninolytic fungi and bacteria is based on the low substrate specificity of their oxidative enzymes, and especially in the discovery of low molecular weight and high redox potential oxidizing agents, which significantly increase the range of compounds susceptible to degradation. These agents include hydroxyl radicals (OH) generated through quinones redox cycling. Among the ligninolytic microorganisms, OH production has been demonstrated in white and brown-rot fungi. Basically quinone redox cycling consists in intracellular quinone reduction followed by the secretion and further oxidation of the generated hydroquinone into a semiquinone. In white-rot fungi this reaction is catalysed by ligninolytic enzymes such as laccases [4]. Autoxidation of semiquinones radicals closes the cycle leading to the production of superoxide radical anion which dissipates to $H_2O_2$. If this cycle is carried out in the presence of $Fe^{3+}$, its reduction by both superoxide and semiquinone generates $OH^-$ via Fenton reaction.

For years, our research group has been working in the characterization of the enzymatic complex involved in the lignocellulose degradation in streptomycetes. As far as ligninolytic enzymes such as peroxidases [5, 6] and more recently laccases [1, 2] have been described in different Streptomyces strains, the interest to know if laccases were able to enhance its oxidative potential of Streptomyces cyaneus CECT 3335 throughout the production of hydroxyl radical via quinone redox cycling has emerged.

Results obtained in this study showed the first evidence on the production of extracellular hydroxyl radicals (OH) via quinone redox cycling by a ligninolytic bacteria. It has been found that the incubation of S. cyaneus at pH 5 with lignin peroxidase and 2,6-dimethoxybenzoquinone resulted in the production of $OH^-$ on a constant basis. The redox cycle is catalysed by a still uncharacterised intracellular system, which reduces quinone to hydroquinone, and the ligninolytic enzyme lactase, converting hydroquinone into semiquinone. Oxidation of the semiquinone by $O_2$ and $Fe^{3+}$ regenerates the quinone, with the concomitant production of superoxide anion radical ($O_2^-$ and $Fe^{2+}$). Then, after $O_2^-$ dismutation, the $H_2O_2$ generated is decomposed to $OH^-$ by $Fe^{2+}$ (Fenton reaction). Production of OH by this mechanism has been shown to be operative for several hours, being attributable to the quinone reductase activity only during the exponential phase of growth. Nevertheless, the OH production can be improved by raising the pH of the incubation to values enabling hydroquinone autoxidation. Based on the strong oxygen reducing power of OH, these findings have led us to evaluate how much the stimulation of OH production in S. cyaneus could increase its biodegradative capacity. Using benzene as a xenobiotic model compound, our current results are revealing that there is a good correlation between OH levels and benzene degradation rate. Finally, 2 days old mycelium of S. cyaneus together with 2,6-dimethoxybenzoquinone and $Fe^{2+}$ was assayed to degrade 500 $\mu M$ of benzene, toluene, ethylbenzene and the three xylene isomers. The degradation rate achieved against all BTEX after 4 h incubation was between 50-60% compared with a control without quinone and $Fe^{3+}$.

References

Keywords: Quinone redox cycling, Streptomycetes, laccase, BTEx degradation

Fed-batch and repeated fed-batch cultures of Candida sp. in an airlift bioreactor for the removal of Cr(VI) from aqueous solutions

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Hexavalent chromium compounds [Cr(VI)], mainly chromates and dichromates, are widely used in many industrial processes, including manufacturing of metallic alloys (the most important use of chromium), chrome leather tanning, metal cleaning processing, wood preservation, ceramics, pyrotechnics, electronics, and so on. The large volumes of contaminated wastewater generated from those industrial processes and their mishandling have caused contamination of water bodies, soils and sediments. Some microbiological methods have been proposed to remove Cr(VI) from wastewaters since they are considered effective and economically feasible alternatives to conventional treatment methods. However, some microorganisms lose viability and Cr(VI)-removing capacity in the presence of high concentrations of Cr(VI), which further complicates the biological treatment of Cr(VI)-contaminated wastewater. Therefore, it is important to explore some strategies that could allow to diminish the toxic effects of Cr(VI) on microorganisms. One potential alternative could be the use of reaction systems in which low Cr(VI) concentrations in the culture medium are maintained, such as fed-batch and repeated fed-batch cultures.

The main aim of this work was to examine the ability of Candida sp. to remove Cr(VI) in fed-batch and repeated fed-batch cultures. The fed-batch and repeated fed-batch cultures were conducted in an airlift bioreactor with a multi-perforated concentric tube. The fed-batch cultures were initiated with a 2.5-L batch culture and when the Cr(VI) had been almost completely removed, the continuous addition of culture medium containing 1.5 mM Cr(VI) was begun. After reaching a total liquid volume of 3.7 L, the bioreactor was drained to a medium volume of 2.5 L. Thereafter, the supply flow of fresh culture medium was maintained constant until a total liquid volume of 3.7 - 4 L was reached (repeated fed-batch culture). Two feeding rates of Cr(VI) were tested in both the fed-batch and repeated fed-batch cultures, 0.514 and 1.05 mg Cr(VI) h⁻¹. Experiments were carried out at room temperature (24 ± 1 °C) and the air flow rate was maintained at 0.5 vvm.

Results showed that the total cell mass attained in the fed-batch cultures was higher than the obtained in the repeated fed-batch cultures. The highest efficiencies of Cr(VI) removal were achieved when a Cr(VI) feeding rate of 0.514 mg h⁻¹ and it was obtained in the fed-batch culture carried out with a Cr(VI) feeding rate of 1.05 mg h⁻¹. The glucose consumption efficiency increased as the incubation time progressed.

The above results suggest that the fed-batch process could be an effective biological method for the detoxification of Cr(VI). The volumetric rate of Cr(VI) reduction exhibited by Candida sp. in fed-batch and repeated fed-batch cultures decreased as the incubation time increased. Moreover, the volumetric Cr(VI) reduction rates were higher in the fed-batch cultures than in the repeated fed-batch ones. The highest volumetric rate of Cr(VI) reduction was 0.226 mg L⁻¹ h⁻¹ and it was obtained in the fed-batch culture carried out with a Cr(VI) feeding rate of 1.05 mg h⁻¹.

The above results suggest that the fed-batch process could be an effective biological method for the detoxification of Cr(VI)-laden wastewaters.

Keywords: Cr(VI) removal; Candida; fed-batch culture; repeated fed-batch culture
Functional expression and substrate specificity of three ring-hydroxylating dioxygenases from the PAH-degrading strain Mycobacterium 6PY1

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Contamination of soil and sediments by polymeric aromatic hydrocarbons (PAHs) is widespread. The persistence of toxic pollutants, especially PAHs comprising 4-5 cycles, may pose health problems, thus justifying the implementation of bioremediation strategies to treat polluted sites, based on the ability of certain microorganisms to degrade PAHs. Increasing evidence suggests that sphingomonads and Mycobacterium species mainly contribute to PAH degradation thanks to dedicated catalytic enzymes. Ring-hydroxylating dioxygenases (RHDs), which catalyze the initial attack of various aromatic hydrocarbons, play a critical role in the oxidative degradation of PAHs. In previous work, we showed that in Sphingomonas CHY-1 a single RHD endowed with an exceptionally broad substrate specificity, catalyzed the dioxygenation of 9 of the 16 priority PAHs including benzo[a]pyrene (1–3). Here, we investigated the properties of three RHDs found in the pyrene-degrading strain Mycobacterium 6PY1. The oxygenase component of two RHDs (Pdo1 and Pdo2) were previously identified in this strain by proteomic analysis of pyrene-induced proteins (4), but their catalytic activity could not be studied because we lacked an appropriate expression system. In addition, associated electron carriers had not been found.

Two sets of genes encoding additional oxygenase component of RHDs, called Pdo3 and Pdo4, have been identified in the genome of strain 6PY1. The deduced sequences of the Pdo3 and Pdo4 polypeptides were highly similar to each other and closely related to Pdo1. Recombinant expression of Pdo1, Pdo2 and Pdo3 was examined in different E. coli host cells using vectors having either a P15 or Ppromoter. Best results in terms of enzyme activity were obtained when expression was driven from the Ppromoter, irrespective of the host cells used.

Besides, a pair of genes coding for a reductase and a ferredoxin, were found close to catalytic genes related to PAH metabolism. Due to the relatively high frequency of rare codons in their sequence, successful over-expression of these genes in E. coli required that a strain supplying rare tRNAs (Rosetta) be used. The ferredoxin was purified as a 84-kDa monomeric protein containing a [3Fe-4S] cluster, in contrast with most RHD-associated ferredoxins which contain a [2Fe-2S] cluster.

When Pdo1 and Pdo2 were separately co-expressed with the ferredoxin and the reductase in E. coli, the enzyme activity (phenanthrene as substrate) was increased 10-fold and 2-fold, respectively. Both oxygenases used phenanthrene as best substrate, but Pdo2 showed 3- to 4-fold higher specific activity. In addition, while Pdo2 produced 1,4-phenanthrene dihydrodiol as single product, the Pdo1 enzyme also produced the 9,10-isomer. Pdo1 also converted pyrene, fluoranthene and anthracene to corresponding dihydrodiols but at slower rates. On the other hand, Pdo2 catalyzed the oxygenation of naphthalene, anthracene and biphenyl, but showed negligible activity with pyrene and fluoranthene. The Pdo1 exhibited a substrate selectivity similar to that of Pdo1. Hence, utilization of PAHs by Mycobacterium 6PY1 involves at least two types of RHDs with narrow substrate ranges. The presence of multiple copies of Pdo1-like oxygenases, if simultaneously expressed in bacterial cells, might be a means to cope with relatively low specific activity of this oxygenase for pyrene and other 4-ring PAHs. Our data have general relevance given that many Mycobacterium strains found on PAH-contaminated sites have genes coding similar RHDs.

Keywords: dioxygenases; ring hydroxylation; PAH biodegradation; ferredoxin, Mycobacterium

Fungal strains capable to use polyurethane as sole carbon source

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Polyurethanes (PU) are plastics whose are used as raw material in several industries. This xenobiotic substance has been used as adhesive, flexible foam, elastomer, covering, etc. It is made from a diol and a diisocyanate and it is toxic pollutants, especially PAHs comprising 4-5 cycles, may pose health problems, thus justifying the implementation of bioremediation strategies to treat polluted sites, based on the ability of certain microorganisms to degrade PAHs. Increasing evidence suggests that sphingomonads and Mycobacterium species mainly contribute to PAH degradation thanks to dedicated catalytic enzymes. Ring-hydroxylating dioxygenases (RHDs), which catalyze the initial attack of various aromatic hydrocarbons, play a critical role in the oxidative degradation of PAHs. In previous work, we showed that in Sphingomonas CHY-1 a single RHD endowed with an exceptionally broad substrate specificity, catalyzed the dioxygenation of 9 of the 16 priority PAHs including benzo[a]pyrene (1–3). Here, we investigated the properties of three RHDs found in the pyrene-degrading strain Mycobacterium 6PY1. The oxygenase component of two RHDs (Pdo1 and Pdo2) were previously identified in this strain by proteomic analysis of pyrene-induced proteins (4), but their catalytic activity could not be studied because we lacked an appropriate expression system. In addition, associated electron carriers had not been found.

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When Pdo1 and Pdo2 were separately co-expressed with the ferredoxin and the reductase in E. coli, the enzyme activity (phenanthrene as substrate) was increased 10-fold and 2-fold, respectively. Both oxygenases used phenanthrene as best substrate, but Pdo2 showed 3- to 4-fold higher specific activity. In addition, while Pdo2 produced 1,4-phenanthrene dihydrodiol as single product, the Pdo1 enzyme also produced the 9,10-isomer. Pdo1 also converted pyrene, fluoranthene and anthracene to corresponding dihydrodiols but at slower rates. On the other hand, Pdo2 catalyzed the oxygenation of naphthalene, anthracene and biphenyl, but showed negligible activity with pyrene and fluoranthene. The Pdo3 exhibited a substrate selectivity similar to that of Pdo1. Hence, utilization of PAHs by Mycobacterium 6PY1 involves at least two types of RHDs with narrow substrate ranges. The presence of multiple copies of Pdo1-like oxygenases, if simultaneously expressed in bacterial cells, might be a means to cope with relatively low specific activity of this oxygenase for pyrene and other 4-ring PAHs. Our data have general relevance given that many Mycobacterium strains found on PAH-contaminated sites have genes coding similar RHDs.

Keywords: dioxygenases; ring hydroxylation; PAH biodegradation; ferredoxin, Mycobacterium

References
Genetic engineering of *Cupriavidus metallidurans* CH34 for bioremediation of heavy metals in wastewater.

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This work describes the targeting of a synthetic metal-chelating protein to the cell surface of the heavy metal resistant *Cupriavidus metallidurans* CH34 bacterial strain. This bacterium is adapted to thrive in soils highly polluted with metal ions and, thus, has been considered a suitable candidate for bioremediation. For this purpose, an analog gene of the natural phytochelatin (Glu-Cys)nGly with n=20, EC20sp, was synthesized in vitro and fused to the autotransporter β-domain coding sequence of the IgA protease of *Neisseria gonorrhoeae*, which successfully targeted the hybrid protein towards the bacterial outer membrane. The translocation, surface display, and functionality of the EC20sp-β-domain protein were initially demonstrated in *Escherichia coli* upon insertion of the fused protein under control of the plcA promoter. As for the *C. metallidurans* CH34 strain, the EC20sp-β-domain gene fusion was placed under the control of a new heterologous promoter which allowed strong levels of basal expression, that were even stronger in the presence of metal ions. The recombinant strain, *C. metallidurans* pCM2, proved to have significantly enhanced ability for immobilizing Cd²⁺, Co²⁺, Cu²⁺, Hg²⁺, Mn⁴⁺, Ni²⁺, Pb²⁺ and Zn²⁺ ions from the external media as compared to the wild type.

**Key words:** Genetic engineering. *Cupriavidus metallidurans* CH34. Bioremediation. Wastewater. Heavy metals. Synthetic phytochelatin.

Growth of *Trametes versicolor* on nitro- and hydroxyl-phenol derivates

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The mycelium of basidiomycete fungus *Trametes versicolor* was earlier shown to grow on phenol as its sole carbon and energy source. The presented research refers to the ability of the strain to degrade and assimilate some nitro- and hydroxyl-phenol derivates.

The culture growth and degradation was carried out in a liquid Czapek salt medium under aerobic condition at pH 6.5 and 28 °C. The medium was supplemented with 0.5 g/l of o-, m-, p- nitrophenol as well as resorcinol, catechol and hydroquinone, respectively. Each of the investigated compounds was added to the medium as a sole source of carbon and energy.

The investigated strain utilized completely 0.5 g/l of hydroxylated phenols for 80-96 hours. The best degradation was observed in the experiments with resorcinol. The most toxic nitrophenols were differently degraded. The best degradation was registered with o-nitrophenol. The strain was able to degrade it completely in 216 hours. The 68% of m-nitrophenol was removed from the medium in 168 hours. After that period of time the strain could not grow. The worst degradation was observed in the experiments with p-nitrophenol. Only 32% were removed from the culture medium in 120 hours and the strain growth was extremely slow.

The dynamics of degradation processes was investigated and characterized by specific growth rate (*μ*ₚₑₒₘ), metabolic coefficient (k) and rate of degradation (Q). The best utilized compounds were used in experiments with the purpose to determine the intracellular activities of the first three enzymes of the phenol catalytical process. The obtained phenol hydroxylase [EC 1.14.13.7] activities in cells cultivated in a medium complemented with resorcinol as a single carbon source were as follow: with phenol as a substrate in the enzyme reaction mixture - 0.2 U/mg protein, with resorcinol as a substrate in the enzyme reaction mixture - 0.34 U/mg protein. In cells cultivated in a medium complemented with o-nitrophenol as a single carbon source the activities of this enzyme were: 0.17 U/mg protein with phenol and 0.15 U/mg protein with o-nitrophenol. In cells cultivated in a medium complemented with o-nitrophenol as a single carbon source the activities of this enzyme were: 0.17 U/mg protein with phenol and 0.15 U/mg protein with o-nitrophenol. In cells cultivated in a medium complemented with o-nitrophenol as a single carbon source the activities of this enzyme were: 0.17 U/mg protein with phenol and 0.15 U/mg protein with o-nitrophenol. In cells cultivated in a medium complemented with p-nitrophenol, respectively. The activities of catechol-1,2-dioxygenase [EC 1.13.11.1] was 0.15 U/mg protein, and 0.12 U/mg protein in cells grown on resorcinol and o-nitrophenol, accordingly. The activities of *cis,cis*-muconate lactonizing enzyme [EC 5.5.1.3] were determined as 0.33 U/mg protein, and 0.34 U/mg protein in cells grown on resorcinol and p-nitrophenol in the same order.

The results from the implemented enzyme analysis are in a good accordance with the obtained kinetic parameters of investigated degradation processes. The other conclusion which could be made is that the substrate specificity of the first enzyme - phenol hydroxylase is decisive for the rate of degradation of the studied toxic aromatic compounds.

**Keywords** *Trametes versicolor*; phenol hydroxylase; catechol-1,2-dioxygenase; *cis,cis*-muconate lactonizing enzyme; nitrophenols; hydroxyphehnols
Hexavalent chromium detoxification of *Bacillus pumilus*-S4, *Pseudomonas doudoroffii*-S5 and *Exiguobacterium*-S8 in association with Hydrophytes

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Three chromium resistant bacterial strains *Bacillus pumilus*-S4, *Pseudomonas doudoroffii*-S5 and *Exiguobacterium*-S8 isolated from chromium contaminated wastewater / soil that could resist very high concentration of K2CrO4 in L. agar (up to 25 mg ml−1) and acetate minimal medium (2 mg ml−1). Strains showed growth at diverse pH and temperatures ranges and could resist multiple heavy metals. It was observed that the %age Cr (VI) reduction of strain *Pseudomonas doudoroffii*-S5 was more (8.27 mg Cr(VI) 24 hours−1) at lower initial K2CrO4 concentration (100 g ml−1) but overall more chromate (28.4 mg Cr (VI) 24 hours−1) was reduced higher initial concentration (1000 g ml−1). Addition of various heavy metals (ZnSO4, CuSO4 and MnSO4 at 50 g ml−1) in the chromium reduction media did not affect significantly on the Cr (VI) reduction potential of these isolates. Cr (VI) removal potential of strains was accelerated in the presence of *Pistia stratiotes* and *Eichornia crassipes*. Both component (Bacteria and plants) support each other growth which results more Cr (VI) detoxification.

Keywords: Chromium, heavy metals, *Bacillus pumilus*, *Pseudomonas doudoroffii*, *Exiguobacterium*, *Eichhornia crassipes*, *Pistia stratiotes*

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High Throughput Analysis of Aromatic Dioxygenase Gene Amplicons from a Tidal Mudflat Using Titanim-Pyrosequencing

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Aromatic dioxygenase from microbial populations in the environments plays an important role for detoxifying polychlorinated biphenyls (PCBs). It is known that anthropogenic aromatics including PCBs affect not only terrestrial environments but also marine environments (i.e. coastal sediments). However, to our knowledge, very little is known about aromatic dioxygenase for degrading biphenyl in marine sediments. In this study, high throughput analysis using titanium pyrosequencing based upon stable isotope probing (SIP) technique was performed in laboratory microcosms amended with biphenyl and sediments collected from a Korea tidal mudflat. Since the amount of 13C-labeled-DNA, which was isolated by SIP, was not sufficient, 13C-labeled-DNA was amplified using Multiple Displacement Amplification (MDA) approach. To obtain a broader range of aromatic dioxygenase genes, genes were amplified using specific primers targeting rieske iron-sulfur protein as a conserved region and amplicons were high throughput sequenced using titanium pyrosequencing. The translation was done on the fixed reading frame after the primers were removed. As delimited by the primer position, the reading frames 3 and -1 were used to translate reads. After removing the reads that contain stop codons in the translated sequences, have ambiguous sequences in the nucleotides, or are shorter than 225 nt, 1,303 reads (among the total 8,162 reads) were identified as aromatic dioxygenase genes. According to results of BLASTX search, a wide range of aromatic dioxygenase genes targeting biphenyl, benzene, dibenzofuran, and naphthalene were detected, and 50% of the detected genes were found to be putative. Further analyses revealed that the diversity of gene-targeted approach was greater than results of microbial based on 16S rRNA and it provided much more information to detect potential biological or ecological functions based on functional gene of degrading PCBs in a tidal mudflat.

Keywords: keyword; Dioxygenase, Metagenomic, Stable Isotope Probing, Pyrosequencing,
Impact of fungus bioaugmentation on diesel-contaminated soil bioremediation by co-composting

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Co-composting was studied as an ex-situ strategy for bioremediation of soils contaminated with gasoline and diesel. Compost from 4-weeks sludge composting was used as co-substrate. The effect of white-rot fungus bioaugmentation by inoculating *Trametes versicolor* was also analyzed.

Co-composting experiments were undertaken for 60 days in 4.5 L reactors, thermally isolated and equipped with on-line temperature monitoring and intermittent aeration system to ensure a high oxygen level and avoid anaerobic conditions. Experimental conditions as co-substrate dose and inoculation procedure were previously adjusted in petri dishes experimental trials. Soil presented a 5% of organic matter and was contaminated with a 3% of a mixture of gasoline and diesel (ratio 1:1).

Three different approaches were tested: co-composting (CC); bioaugmentation by inoculation of *T. versicolor* at initial time and 21 days (BA); and bioaugmentation by inoculation of *T. versicolor* after 21 days of co-composting (after the first initial decomposition phase) (CB). Experiments were undertaken in duplicate.

After 60 days of co-composting a final total petrol hydrocarbon (TPH) reduction around 60% was achieved. The biodegradation level achieved was higher for short chain hydrocarbons, being approximately 94% for C10-C12 fraction, 76% for C12-C16, 60% for C16-C21 and 50% for C21-C30.

Obtained results showed that initial bioaugmentation with *Trametes versicolor* significantly increased initial hydrocarbons degradation rate in all TPH fractions. In general, oxygen consumption was higher in BA reactors. However, bioaugmentation after 21 days of co-composting was not effective, probably due to competition of inoculated fungus with adapted biomass in the composting matrix. In consequence, fungus inoculation should be undertaken at the beginning of the process. After 60 days, lactate levels decreased significantly in all experiments indicating the disappearance of *T. versicolor*. Final TPH levels were similar in all trials. Consequently, initial bioaugmentation is a useful strategy to accelerate the bioremediation process although its effect is negligible in a long term co-composting process.

**Keywords** soil bioremediation; diesel; fungus; bioaugmentation; *Trametes versicolor*; co-composting; compost

Influence of predation by flagellates on the bacterial response to crude oil input in unpolluted oligotrophic and chronically oil-polluted mesotrophic Mediterranean sites.

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The coupling of limitation by resource (bottom-up) and predation (top-down), as well as the history of the polluted site are of great interest to evaluate the potential of petroleum bioremediation by bacteria. Moreover, the influence of petroleum on flagellate diversity as well as on total and metabolically active bacterial diversity has never been integrated in such scenario. These aspects have been studied in mesocosms experiment (75 L) with seawater originated from unpolluted oligotrophic or chronically oil-polluted mesotrophic sites in the Aegean Sea. We found that the ubiquitous *Paraphysomonas foraminifera* is oil-tolerant and an important grazer of bacteria, limiting their growth always <10^7 cells ml^-1. Peaks of bacterial abundance as well as following peaks of flagellate (prey-predator cycle) were higher in oil+nutrients or oil+emulsifier compared to oil or control conditions, where total hydrocarbon biodegradation (especially alkanes) was greater. Changes in both total and active bacterial diversity were faster in the chronically polluted site, resulting in a better biodegradation of the most complex hydrocarbons (pelycyclic aromatic hydrocarbons - PAH). Interestingly, most of the bacterial population selected after nutrient or emulsifier addition were metabolically active, suggesting that biostimulation privileged the emergence of bacterial species adapted to oil.

Such integrative studies have important implication on understanding the impact and efficiency of bioremediation strategies in marine ecosystems.

**Keywords:** Oil Biodegradation; Biostimulation, Bacterial Predation, Diversity
Influence of readily assimilable carbon sources on the phenol degradation by \textit{Trichosporon cutaneum R57} strain

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The aromatic compounds metabolism in particular phenol and its derivatives is a subject of intensive studies in prokaryotes. Nowadays the investigations of different yeast species that metabolize aromatic compounds are of significant scientific interest. \textit{Trichosporon} yeast strains, isolated from various sources, polluted with toxic compounds are among the most studied yeast with respect to aromatic compound biodegradation in eukaryotes. Usually the environmental pollution is a result of different compounds simultaneously.

The object of present investigation is a strain \textit{Trichosporon cutaneum} R 57 able to grow and degrades phenol as a sole carbon and energy source up to 1 g/l in a short period of time (16-18h). The strain is also able to degrade phenol in a very short period of time in rich medium despite the presence of additional carbon source (such as peptone or amino acid).

The aim of the present investigation is to carry out the influence of additional carbon sources such as glucose and acetate on the phenol degradation in \textit{Trichosporon cutaneum} R57 strain. The activity of two key enzymes for catabolism of phenol as phenol hydroxylase [EC 1.14.13.7] and catechol-1,2-dioxygenase [EC 1.13.11.1] were determined in cells grown in a medium Yeast Nitrogen Base without Amino Acids used for analyses of carbon assimilation in yeast strains comprising different mixtures of the mentioned above carbon sources.

The effect of glucose and acetate on the phenol degradation ability of \textit{Trichosporon cutaneum} R57 strain was examined in the condition of batch cultivation. It was established that in YNB w/o AA medium the glucose and phenol assimilation flowed simultaneously so that the assimilation rate of glucose was much higher than that of phenol. Some delay in phenol degradation was observed in the experiments for studying the acetate influence in the medium. In these experiments however the phenol was completely degraded by the time of acetate assimilation.

The results obtained after enzymes analyses in the cells cultivated in the medium with two carbon sources such as phenol and acetate that are known to be utilized by \textit{Trichosporon cutaneum} R57 are of special interests. In our experiments in a culture medium with 0.5 g/l phenol included the presence of acetate (1.8 g/l) did not influence negatively the phenol hydroxylase activity (0.833 U/mg protein), compared to the activity in the same medium without other than phenol carbon source (1.14 U/mg protein), but obviously lowered twice the activity of catechol-1,2-dioxygenase (0.108 U/mg protein and 0.206 U/mg protein respectively).

On the contrary, the influence of glucose presence in the media had much stronger influence on the activity of both investigated enzymes. In a culture medium containing 0.5 g/l phenol and 1.5 g/l glucose the activity of the phenol hydroxylase dropped to 0.225 U/mg protein and catechol-1,2-dioxygenase activity was 0.65 U/mg protein. It should be pointed that some basal activity of both enzymes (3-5 mU/mg protein) was established even in cells cultivated in a medium with glucose as a sole carbon source.

Our results showed that in these experiments the presence of acetate, respectively glucose do not repress and/or inactivate phenol degradation enzymes such as phenol hydroxylase and catechol-1,2 dioxygenase in the investigated \textit{Trichosporon cutaneum} R 57 strain.

On the basis of our previous and recent analyses, the investigated strain could be considered to have a good potential for application in remediation of phenol contaminated environment and improvement of phenol removing treatment of industrial wastewaters.

Keywords: phenol biodegradation; glucose; acetate; phenol hydroxylase; catechol-1,2-dioxygenase; \textit{Trichosporon cutaneum}

Inoculation of selected Rhizobacteria favours plant growth under stress conditions

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Introduction and Experimental Procedures. Many processes in natural soils of warm regions frequently produce saline soils. In these conditions, an inadequate water regime can increase the salinity and represents a significant problem in agriculture. The application of bioinoculants is an environmental and economically viable approach for recovering soils and increasing biomass production. Sunflower is a widely cultivated crop relatively resistant to soil salinity. In this work we studied the inoculation effect of two different rhizobacteria (\textit{Pseudomonas fluorescens} biotype F -white type- and \textit{P. fluorescens} CECT 378 -collection strain-) on the growth of sunflower plants. Furthermore, we assessed the abilities of these bacteria to utilize aminomethylpropane carboxylic acid (ACC) as the sole source of nitrogen, as well as the production of indoleacetic acid (IAA) and siderophores as parameters to characterize the plant-growth promoters. The quantification of IAA production was made following a published protocol [1] and the production of siderophores was determined by a modified method [2]. Sunflower (\textit{Helianthus annuus} L. cv. Sungro 393) seeds were grown in growth chamber conditions [3] during 5 weeks. The inoculation with both bacteria strains was made separately (10^8 cfu/g), every two. Determination of Na+ and K+ content in roots, leaves and stems were determined by atomic absorption spectrophotometry after extraction with acetic acid solution.

Results and discussion. The study of the Na+ tolerance of both bacterial strains in liquid media showed a greater tolerance for the collection strain, just as the production of IAA without triptophan in medium. The microbial production of siderophores is often related with pathogen suppression and iron acquisition. In this way, we have found a positive response in both strains, whose quantification is currently underway at our laboratory. The growth chamber experiment in saline conditions, showed a significant reduction of plant growth, affecting some plant parameters (Table 1).

When NaCl was used to induce salt stress, Na+ content in leaves, stems and roots increased, but the inoculated plants showed less Na+ content in all cases. In salt treatments, the amount of K+ accumulated by leaves and stems was higher than the accumulated amount of Na+, while in roots it was the opposite. The K+/Na+ correlation coefficients in leaves, stems and roots of sunflower plants, showed this decreasing order: leaves > stems > roots. Both bacterial inoculants contribute to increment this coefficient in all parts of the plant.

Conclusions. The inoculation of both bacterial suspensions decreased considerably the levels of accumulated Na+ in all parts of the plant while their K+ content increased, as well as their biomass. Probably, this is due to the stimulation of plant root growth by IAA, better iron status because siderophores production, and to the capability to utilize ACC through ACC-deaminase, thus decreasing ethylene production which implies enhanced root length.

Acknowledgements. Supported by the Fund, “Science Investigation” (Ministry of Education and Science, Bulgaria) and the Univ. of Córdoba (Spain). Keywords: Rhizobacteria, \textit{Pseudomonas fluorescens}, sunflower, stress, salinity.

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Interactions microorganism-salt marsh plants in the presence of Cu and PAHs contamination


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Recovering impacted estuarine environments is a nowadays priority. Remediation methodologies using both microorganisms (bioremediation) and plants (phytoremediation) could be a valid option due to their capability to respond over different contaminants. However, knowledge about the occurrence of antagonisms and synergisms effects among different pollutants and how they influence plant-microorganisms interactions is still scarce. This study aimed to investigate the effect of Cu and PAHs, two common pollutants in estuaries, on salt marsh microbial community, in the presence/absence of Halimiones portulacoides, salt marsh plant by means of sediment and elutriate solution experiments, in controlled environmental conditions. Cu and PAHs concentrations were measured in solutions and in sediments at the beginning and at the end of the experiments. Toxicity was measured using the test ToxScreen. In order to estimate microbial abundance total cell counts (TCC) were enumerated by DAPI and bacterial diversity was characterized by means of automated rRNA intergenic spacer analysis (ARISA).

The chemical analysis showed that none of the contaminants (Cu or PAHs) interfered with each other concentrations solution or sediment. Also, the presence of plants seemed to have no influence on Cu levels. However, the presence of plants clearly interfered with the degradation of higher molecular weight PAHs in sediments, since the pace of biodegradation was lower. In fact, the TCC showed lower microbial numbers in the presence of plants. These findings can reflect a competition between plants and microorganisms for nutrients, and highlights the need of fertilization in order to obtain optimal effects of phytoremediation for this type of compounds. On the other hand, in the absence of plants, Cu displayed higher toxicity when compared to other treatments. This fact can be related to Cu complexation by organic compounds released by the plants. In terms of bacterial diversity, it was observed that plants had also an effect on microbial community but only in control and Cu treatments, whereas the presence of PAHs, alone or in combination with Cu, seemed to overcome the eventual effect induced by the presence of plants.

Keywords: microbial community, plant-microorganisms interactions, salt marshes, Cu, PAHs

Isolation and characterization of Ni resistant endophytic bacteria from Alyssum serpyllifolium and their potential in promoting plant growth and Ni accumulation by host and non-host plants

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Phytoremediation is an environmental friendly, cost-effective and plant-based solution for the remediation of heavy metal-contaminated soils. The benefits of combining endophytic bacteria with hyperaccumulating plants for increased remediation of pollutants have been successfully tried for heavy metal removal from contaminated soils. This study was undertaken to assess whether the Ni resistant-endophytic bacteria with general PGP traits, such as the ability to produce IAA, siderophore and ACCD and solubilize P, promote growth uniformly across hyperaccumulator plant species including non-hosts in Ni contaminated soils. Four Ni resistant-plant growth promoting endophytic bacteria (PGPE) were isolated from the tissues of Alyssum serpyllifolium grown in serpentine soils, Braganca, Portugal and subsequent testing revealed that they all exhibited PGP traits associated with plant growth promotion. The plant growth promoting effects of PGPE on their host A. serpyllifolium and another brassicaceous plant, Brassica juncea were assessed with different concentrations of Ni in phytagar medium. The results indicated that none of the four isolates produced any disease symptoms or abnormalities in both test plants. However, among the strains, A3R3 significantly increased the biomass and Ni content of both plants. Further, a pot experiment was also conducted with Ni supplemented soils using A. serpyllifolium and B. juncea. The inoculation with A3R3 increased the biomass of both test plants. Our observations showed that A3R3 protect the plants against the inhibitory effects of nickel, probably due to the production of IAA, siderophore, ACCD and solubilization of phosphate. In addition, A3R3 significantly increased the Ni concentration in the shoots of A. serpyllifolium and another brassicaceous plant, B. juncea. The analysis of population densities of inoculated A3R3 demonstrated that this strain was able to colonize the root and shoot interior and rhizosphere soil of host and non-host plants. The plant growth promotion and colonization potential of A3R3 in the test plants, suggests that inoculation with metal resistant PGPE might have significant potential to improve phytoremediation efficiency in metal contaminated soils.

Keywords: Endophytic bacteria, Nickel; Phytoextraction; Alyssum serpyllifolium
Isolation and screening of bacteria to decolorize Azo-dyes
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Different bacteria were isolated from textile effluents. Out of the 17 bacterial isolates, three isolates were found to make a clear zone around their colonies on azo-dye supplemented media plates. Further these were screened for azo-dye decoloration under liquid conditions. The isolate 1DD was found capable to decolorize the broth within 16 hour at 37°C under stationary conditions. The isolate can be further exploited for decoloration of textile dye effluent which is the major problem of textile industry as well a threat to aquatic life.

Isolation and selection of phenol-degrading bacteria from the wastewater-contaminated soils in Iran
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Bioaugmentation can be assumed as a promising technology for biodegradation of organic substances in wastewater treatment (WWT) systems. The technology should be effective in dealing with contamination to hazardous and toxic chemicals such as phenol.

Phenol is now one of the most common toxic environmental pollutants, which mainly originates from industrial processes and its bioremediation is very important to meet the environmental regulations.

The aim of this study at first was to examine the bioremediation potential of aerobic WWT system by screening naturally occurring population of phenol degrading bacteria. Spreading a number of samples of the activated sludge on standard agar plates, as well as pretreatment in enriched broth, resulted in isolation of 8 phenol resistant bacterial strains out of 324 different isolates, but poor phenol degrading capacity was observed. The preliminary results addressed to requirement of active exogenous microorganisms.

To screen high strength phenol degrading bacteria, we sampled wastewater-contaminated soils in an industrial region of Tehran province, Iran, and a phenol-degrading bacterium designated as SKO-1 was isolated from these soils by direct spreading plate method, in order to avoid biodiversity alteration during the course of enrichment.

This gram positive, non-motile, non-spore-forming and rod shaped bacterium was able to utilize phenol, as the sole carbon source in the mineral medium with the phenol concentration varying from 100 to 1000mg/l. The cultures were grown aerobically in 250 ml flasks, the temperature in all the batch experiments was maintained at 30±0.1 °C and the shaker speed was maintained at 130 rpm. The influence of the adaptation of the bacterium to the substrate was studied as well. The well-acclimatized culture of SKO-1 degraded the initial phenol concentration of 1000 mg/l completely in less than 72 hours.

Bioaugmentation using various bacterial strains has proved to be a promising option for the clean-up of polluted sites and it can be a suitable tool for biotransformation and biodegradation of many recalcitrant organic compounds as well as phenol.

Keywords: Bioaugmentation; biodegrading bacteria; biotransformation; phenol

This work, as a part of the project (No.0890188703), is supported by Petrochemical Research and Technology Co. in Iran.
Microbes and their contribution in environmental sustenance: Multipotent microbes from East Calcutta Wetland

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This study put forward the bioremedial potential of novel isolates from a wetland ecosystem of Kolkata viz. East Calcutta Wetland (ECW). This wetland ecosystem spans an area of 12,500 hectares and it acts as the dumping ground for the mega city. But the significant fact remains that this region also acts as a resource recovery system where waste is recycled and used in production of commercial products like paddy, vegetables as well as fish. The diversity of the microbial resource of this region was depicted by the culture independent approach and this study reveals presence of significant groups like Acinetobacter, Actinobacteria, Proteobacteria and Firmicutes. The current study aims at culture dependent isolation of potential microbes followed by their complete characterization and extensive study on their bioremedial potential which would cover up remediation of heavy metals as well as crude oil.

The culture based technique resulted in nine pure bacterial isolates from soil and water samples collected from different sites of ECW produced. The isolates were characterized based on their morphological, physiological and biochemical features. The 16S rDNA homology based molecular identification indicates the bacterial isolates as belonging to two different genus, Acinetobacter and Pseudomonas.

All the isolates were found to tolerate heavy metals like nickel (Ni), copper (Cu), silver (Ag), aluminium (Al), iron (Fe), chromium (Cr), lead (Pb) upto different extent. Energy Dispersive Xray Fluorescence (EDXR) analysis provided the extent of metal accumulation where as Transmission Electron Microscopy revealed the localization of metal inside the cell. The cellular response towards the metal induced stress was detected by Scanning Electron Microscopy and the significant changes observed were shortening of cell, cell elongation or the development of extracellular matrix. Presence of SOD gene was detected by using universal primers and its presence also supported the cellular defense mechanism.

The isolates were found to grow in vegetative oils like coconut oil as well as mineral oils like diesel, mobil and burnt mobil. The Acinetobacter isolates showed more than 65% degradation of lubricants like mobil and burnt mobil in a span of 72 hours.

The significant outcome of this study would be the employment of these isolates in remediation of environment co contaminated with metals as well as with oil spills.

Keywords East Calcutta Wetland, Bioremediation, Heavy metals, Crude oil.
Monitoring of oil-degrading bacteria during bioremediation by foodborn compost

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Oil spills is one of the main pollution in soil, groundwater, and marine environments. For successful bioremediation, it is essential to understand the dynamics of microbial population responsible for the degradation of the target pollutants at the remediation sites. We focused on foodborn compost as the bioremediation promoter because it is rich in nutrients and in diverse microorganisms, and the use of compost in bioremediation supports recycling.

In this study, we investigated the effects of foodborn compost on the fate of total petroleum hydrocarbons (TPH) and oil-degrading bacteria during bioremediation.

We tested four bioremediation promoters—nutrients, mushroom, biofertilizer, and foodborn compost—and no promoter (control). We collected samples periodically and determined TPH concentration. During 89 days, the TPH concentration was reduced from an initial 4,700 ppm to 4,300 ppm in the control, to 2,700 ppm with nutrients, to 2,800 ppm with mushroom, and to 2,850 ppm with biofertilizer. In contrast, it was initially increased to 8,300 ppm with compost, and was then reduced to 2,400 ppm during 74 days, showing the highest degradation rate among all treatments. In addition, the compost removed the oil stench at the TPH concentration reached 2,500 ppm.

To investigate the effects of each treatment on the total number of bacterial cells, we quantified 16S rDNA by real-time PCR. The number of cells in the control changed little, but those in the other treatments increased quickly.

To monitor the behaviors of oil-degrading bacteria, we designed PCR primers to detect alkB, alkM, C12O gene, and C23O gene. After verification of their availability for soil samples, these genes were quantified by real-time PCR. Bacteria possessing alkB increased in all treatments, including the control. Bacteria possessing alkM were increased in all promoter treatments but not the control. Catechol-degrading bacteria possessing C23O gene were not so many in the nutrients and mushroom treatments relatively, but those possessing C12O gene concerned in degradation of same substance proliferated in these treatments, indicating that the bacterial structures depended on the promoters added. C23O gene was detected from the start, but C12O gene became detectable in the late phase of bioremediation.

These results suggest that oil-degrading bacteria were increased most by the addition of foodborn compost, which led to a remarkable decrease of TPH concentration.

Keywords: bioremediation; oil; compost; real-time PCR

NAA for studying effects of potentially toxic metals (Cr, Hg) on Arthrobacter globiformis

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Bacterial reduction and detoxification of potentially toxic metals is one of most promising strategies for the bioremediation of the contaminated environmental media. In our previous studies we have established that Gram-positive bacterial strain Arthrobacter globiformis isolated from polluted basalts of Georgia can reduce and detoxify of Cr(VI) with high efficiency [1]. In the present investigation instrumental neutron activation analysis (INAA) was applied to study (1) accumulation of Cr(VI) in A. globiformis in the presence of Hg(II); (2) accumulation of Hg(II) in bacterial cells; and (3) effects of Hg (II) and mixture of Cr(VI) -Hg(II) on the elemental composition of bacteria. Our experiments were focused on the dose-dependent effects of Cr (VI) and Hg(II). Cr(VI) as [K2Cr2O7] and Hg(II) as [Hg(NO3)2⋅H2O] were added to the bacterial cell cultures at an early stationary phase of their growth. Two sets of experiments were performed. In the first set the concentration of Hg(II) varied within the range of 50–5000 μg/L. In the second set a 500 μg/L concentration of Hg(II) was added to the bacterial cells at each given concentration of Cr(VI) within the range of 50–1000 mg/L. According to the results obtained, the dose-dependent character of Cr(VI) accumulation by tested bacterial strain was not significantly affected by the presence of Hg(II). Accumulation of Hg(II), similar to Cr(VI) accumulation, follows well the Langmuir-Freundlich model. Besides, NAA measurements showed the increased content of Fe in bacteria under Hg and Cr action, suggesting that Fe-containing biomolecules play decisive role in detoxifying of heavy metals by A. globiformis. The concentration of 5000 μg/L of Hg(II) was found to be critical for A. globiformis. At this concentration of Hg(II) the concentrations of both essential (Na, Mg, Al, Cl, K, Mn, Zn) and some non-essential elements (Rb, Sb, Sc, As) changed drastically along with decrease of the biomass of bacteria by factor of 2. One may assume that under this concentration loading of Hg(II) the structure of bacterial cell wall was destroyed.

Keywords: Arthrobacter globiformis, NAA, Cr(VI), Hg(II), accumulation


Acknowledgement: We are grateful to STCU for their support (Grant #4330)
New proteins expression by PGPR *Pseudomonas fluorescens* under arsenic-induced stress conditions

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**Introduction and Experimental Procedures.** Some *Pseudomonas* strains promote plant growth and heavy metal accumulation in contaminated soils and they possess an efficient system to survive in abiotic stress conditions [1]. Proteomics is one of the best strategies used to reveal the dynamic expressions of whole proteins in cells and their interactions. The aim of this work was to study the protein profile changes and the differentially expressed proteins in *P. fluorescens* biotype F (tolerant to arsenic) and *Pseudomonas fluorescens* CECT 378 (sensitive), exposed to sodium arsenate. Both strains were growth separately in the presence or absence of 1000 ppm As (Na3H2AsO4), till reaching medium stationary phase. After centrifugation and washing, the whole cells extracts were obtained by resuspending the pellet in lysis buffer and ultrasonication. The quantification was made using Bradford method followed by loading of the protein sample on the 17cm IPG strip pH 4-7. IEF was performed in focusing tray by rehydration and changing the tension of the linear current. After equilibration with DTT and iodoacetamide buffers, SDS-PAGE was performed. All the experiments were repeated twice. Gels were stained with 1% Coomassie Brilliant Blue G250 to visualize the proteins [2] and they were scanned and analyzed using PD-Quest software version 7.1.0 (Bio-Rad). The differences between selected spots in control and arsenic treatments were considered to be significant by ANOVA (*p*<0.05) (Statistix 8.0). Furthermore, tryptic digestion of protein of interest were picked automatically using Investigator ProPac station (Genomic Solutions). Resulted peptides of the trypsin digestion had been purified and after corystallization the samples were analyzed in a range between 800 and 4000Da using mass spectrometry MALDI-TOF/TOF to obtain a peptide fingerprint (MS) in a spectrometry 4700 Proteomics Analyzer (Applied Bioystems, USA). Moreover, the protein identification was realized using Mascot (MatrixScience, UK) in MSDB database.

**Results and discussion.** The mechanisms of tolerance are of essential interest to discover how bacteria survive the extreme conditions. The pattern of protein separation expressed using two-dimensional PAGE was consistent in all the gels. Comparing the treatment for each strain with corresponding control, the appearance of a total of 9 differentially expressed and statistically significant protein spots in *P. fluorescens* biotype F and 7 in *P. fluorescens* CECT 378 after exposure to 1000 ppm of arsenic, had been observed. In tolerant strain, in presence of arsenic, were found 4 newly appeared, 4 upregulated and 3 downregulated spots, comparing with the corresponding control. On the other hand, from the 7 differentially expressed spots in the non-tolerant strain, 1 was newly appeared, 3 upregulated and 3 downregulated.

The most significant findings in case of tolerant strains, in the presence of As, was the appearance of enzyme arsenate reductase (spot 5). This enzyme catalyses the conversion of arsenate in arsenite, that is a more toxic substance. When tolerant bacterial cell detects the arsenite, it is extruded out of the cell through the membrane system. On the other hand, the unique appeared protein, in case of the sensitive strain, is tyrosine phosphatase (spot 5).

**Conclusions.** The *Pseudomonas fluorescens* strains showed important changes when grown under arsenic stress conditions; changes that were statistically significant. The tolerant strain showed 4 newly appeared spots, while the sensitive presented just 1 spot. All of these spots have a low molecular weight. Thus, we propose arsenate reductase to be the main determinant of tolerance of wild-type *P. fluorescens*.

**Acknowledgements.** This work was supported by the Spanish Agency for International Cooperation (AECI).

**References**


**Keywords:** *Pseudomonas fluorescens*, protein profile, PGPR, abiotic stress, As tolerance, MALDI-TOF

Ni, Pb and Cd tolerance by *Phanerochaete chrysosporium* in industrial wastewater

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This microorganism has been considered not only for use in bioleaching of metals but also as accumulators of metals from wastewaters. On the other hand, their ligninolitic enzymes can be implicated in the mechanisms tolerance with the melanin production.

The effect of cadmium sulphate, nickel chloride, and lead acetate on the growth and tolerance of *P. chrysosporium* in synthetic wastewater at different concentrations of these heavy metals was investd. According to results *P. chrysosporium* was tolerant strain with IMC of 300, 50, 4500 mg/L for Cd, Ni and Pb. The removal of three metals by free and immobilized biomass were investigated using the 300 mg/L for each metal and the mixture in aqueous solutions by 5 d at 30°C, observing that the free biomass and the immobilized biomass removed 74%, 98%, 57%, 0%, 93% and 83% of Cd, Pb and Ni respectively. The biosorption of the metals on the fungal wall were analyzed by SEM and EDX demonstrating that the chitin was a good bioadsorbent with high affinity for Pb. The enzymatic activity MnP only was detected in presence of Pb with 0.018 U/L. The LiP activity was expressed in presence of Cd, Ni and in the mixture with 46, 37 and 22 U/L.

In conclusions *P. chrysosporium* is a hypertolerant strain with biotechnological application for heavy metal removal in industrial wastewater.
Novel microbes from East Calcutta Wetland: Implications for environmental sustenance

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The present study was the exploitation of rich microbial resource of a wetland ecosystem of Kolkata viz. East Calcutta Wetland (ECW). It is worth mentioning that this wetland region of about 12,500 hectares acts as the dumping ground for the mega city but at the same hand it also acts as a resource recovery system where waste is recycled and used in production of paddy, vegetables as well as fish. The culture independent studies had revealed the existence of diverse bacterial population in the wetland ecosystem and indicate presence of significant groups like Acinetobacter, Actinobacteria, Proteobacteria and Firmicutes. With this preexisting data, the objectives of the current work were the following (a) culture dependent isolation of potential microbes followed by their complete morphological, physiological, biochemical and molecular characterization, (b) to investigate their heavy metal uptake as well as crude oil degradation efficiency and (c) the assessment of these isolates from a commercial viewpoint keeping in view their ability to produce extracellular enzymes like lipase and protease.

Microbial enrichments from soil and water samples collected from different sites of ECW produced nine pure bacterial isolates. Preliminary characterization was based on the morphological, biochemical and physiological properties. 16S rDNA homology revealed the molecular characterization and the bacterial isolates were identified as belonging to two different genus, Acinetobacter and Pseudomonas.

All the isolates were found to tolerate heavy metals like nickel (Ni), copper (Cu), silver (Ag), aluminium (Al), iron (Fe), chromium (Cr), lead (Pb) up to different extent and EDTA analysis indicates maximum accumulation found in case of Pb salt. Transmission Electron Microscopy reveals the throughout intracellular accumulation of metals making them good candidate for concentration of toxic metal. Scanning Electron Microscopy depicts the cellular response towards metal induced stress either in form of cell shortening, cell elongation or the development of extracellular matrix to cause minimization of metal microbe interaction. Detection of SOD gene further strengthened the cellular defense mechanism.

The isolates were found to utilize vegetative oils like coconut oil as well as mineral oils like diesel, mobil and burnt mobil as principal carbon sources for growth. The Acinetobacter isolates showed more than 65% degradation of lubricants like burnt mobil and mobil in a span of 72 hours.

Lipase enzyme from one of the isolate was used as an additive to detergent and provided efficient wash performance in multipurpose functions like cleaning of glasswares, clothes, utensils, sinks etc. A market survey indicated wide acceptability of the detergent with enzyme additive within different socio economic population. The extracellular enzymes protease and lipase from one of the isolates were tried out as dehairing agents for the treatment of goat hide. Washing with the enzyme resulted in better dehairing, as soaking and degreasing were found to be enhanced on application of enzyme as compared to the conventional process.

The main aim of this work was to study the effect of different conditions such as pH, initial metal concentration, contact time, and temperature on the capacity of untreated and acetone-pretreated R. glutinis biomass to biosorb Ni(II) ions from aqueous solutions. Furthermore, various kinetic and isotherm models were tested to describe the kinetics and Ni(II) biosorption data.

Results showed that the capacity and initial volumetric rate of Ni(II) biosorption of untreated and pretreated R. glutinis biomass increased with time. The biosorption capacity of the pretreated yeast biomass was 17% higher than that of the untreated one, so the pretreated biomass was chosen to carry out later studies.

The experiments performed at different initial Ni(II) concentrations showed that the Ni(II) biosorption capacity increased with the contact time increased, until it reached a constant value which was dependent on the contact time and the initial Ni(II) concentration. The maximum Ni(II) biosorption capacity of pretreated R. glutinis biomass was about 40.22 mg g⁻¹. Among the two-parameter models tested (Langmuir, Freundlich, Temkin, Flory-Huggins and Dubinin-Radushkevich) to describe the equilibrium between the Ni(II) sorbed onto the pretreated yeast biomass and Ni(II) ions in the solution, the best fit was produced by the Langmuir model (r² = 0.99). The maximum Ni(II) biosorption capacity predicted by the Langmuir model (41.67 mg g⁻¹) was very close to that obtained experimentally.

The experimental data were analyzed using two adsorption kinetic models (the pseudo-first- and the pseudo-second-order kinetic equations) to determine the best fit equation for the biosorption of Ni(II) ions onto pretreated R. glutinis biomass. Results showed that the pseudo-second-order kinetic model provided the best fit predicted data with experimental results, which suggests that chemisorption could be the rate-limiting step. From the data obtained at different temperatures, the thermodynamic constants of biosorption (activation energy, enthalpy and entropy) were also evaluated.

Keywords: biosorption, nickel, Rhodotorula glutinis, kinetic models, equilibrium models.
Plant-microorganisms associations in salt marshes: influence on hydrocarbon degradation

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Estuaries are often considered sinks for contaminants, (e.g. hydrocarbons), receiving important anthropogenic inputs from the upstream catchments and from metropolitan areas and industries located on or near those areas. Bioremediation is potentially less damaging and cost effective method for the recovery of contaminated areas compared to alternatives such as soil washing, incineration or disposal to landfill. Soil/sediment microorganisms can be stimulated by plant root exudates and the presence of plants can enhance the potential of microorganisms for bioremediation of soils/sediments contaminated with organic contaminants, like hydrocarbons, by providing specific microenvironments for pollutant-degrading microorganisms.

The aim of this work was to investigate the effect of several salt marsh plants root (Juncus maritimus, Phragmites australis, Triglochin stricta and Spartina patens) commonly found in Atlantic temperate estuaries on the hydrocarbons degradation. The study was carried out in River Lima Estuary (NW Portugal) lower and middle stretches, an urban-industrialized estuary with a large salt marsh area, which is the end member of an international waterbody.

In order to estimate microbial abundance, total cell counts (TCC) of sediment samples were enumerated by DAPI. Culturable hydrocarbon degraders were determined using a modified most probable number (MPN) protocol. For hydrocarbon concentration analysis dry sediment samples were extracted either with acetone or with hexane:acetone (1:1) using ultrasonic extraction or microwave assisted extraction. Extracts were fractioned with Biorbul. Aliphatic hydrocarbons were determined by gas chromatography with flame ionisation detection (GC/FID) and the PAHs were analysed by gas chromatography with mass spectrometry (GC/MS).

The results of the total microbial abundance did not show important differences between sampling sites, between sediments (uncolonized by plants) and rhizosediments (colonized by plants) or between rhizosediments of the different plants. However, in what the hydrocarbon degrader microorganism abundance is concerned, important differences were observed between samples and locations. Comparing the different sampling sites, higher numbers of hydrocarbon degraders were registered in the upper station of the middle estuary. Also it must be noted that, in general, higher numbers of hydrocarbon degraders were observed in sediments colonized by plants (rhizosediments) than in uncolonized sediments. Finally, there were observed differences in numbers of hydrocarbon degraders between the rhizosediments of the different plants collected at the same site (Juncus maritimus < Phragmites australis < Triglochin stricta).

These results show that salt marsh plants can have an important positive influence by enhancing the microbial communities responsible for the hydrocarbon degradation, and that this influence can vary with the plant-microorganisms associations.

This work was partially funded by Fundação para a Ciência e Tecnologia, Portugal, through fellowships awarded to H. Ribeiro (SFRH/BPD/47631/2008).

Keywords: Bioremediation; hydrocarbons; estuary, salt marsh

Bioremediation; hydrocarbons; estuary, salt marsh

References

Preferential utilization of aromatics: modulation of glucose transport proteins in Pseudomonas putida CSV86

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Pseudomonas putida CSV86 utilizes naphthalene, methyl-naphthalene, benzy alcohol, salicylate, benzoate, and hydroxybenzyl alcohol as the sole source of carbon and energy. Growth pattern, enzyme activity profiles and oxygen uptake studies suggests that the organism prefers aromatics over glucose when provided as double carbon source. When glucose grown mid log-phase cells were supplemented with aromatics, the enzymes responsible for aromatic degradation were induced within 30 min. The organism co-metabolizes aromatics and organic acids suggesting that aromatic utilization was not repressed by organic acids as reported for other strains. Metabolism studies suggested that CSV86 utilizes glucose by the indirect phosphorylative pathway. Uptake studies showed that the 14C-glucose uptake was by active transport, inducible and suppressed when the cells were grown in the presence of aromatics or organic acids. Periplasmic-protein profile of cells grown on double carbon source showed a distinct induction of a 43 kDa protein in the second log phase when glucose was being utilized. 2D-protein gel followed by LC-MS/MS analysis showed that the 43 kDa protein (pl 6.5) had sequence similar to ABC transporter protein (mol. wt 45 kDa, pl 6.15) of Pseudomonas putida KT2440. Purification of this protein by gel filtration chromatography and glucose binding assays suggested it to be a glucose binding periplasmic space protein. Aromatic compounds or organic acids were not able to inhibit binding of glucose to the purified protein which rules out the possibility of direct modulation of its glucose binding activity. However, periplasmic protein fractions obtained from naphthalene- or succinate-spiked cells showed immediate inhibition of glucose binding activity, with no significant change in the levels of the 43 kDa protein on SDS-PAGE. This observation suggests that there may be an involvement of a second protein, probably from the periplasmic space, that binds to aromatics or organic acid, which in turn interacts with the 43-kDa periplasmic space protein to inhibit its glucose binding activity. Besides periplasmic protein, a 40 kDa outer membrane protein also showed induction when grown on glucose and suppression when grown on succinate and aromatics. 2-D gel, LC-MS/MS and sequence analysis reveals that the protein has sequence similarity with OprB reported from other P. putida. This protein was purified and characterized. Heat modifiability, [14C]glucose binding, liposomes swelling assay and channel conductance studies indicates that the purified protein was a trimeric channel forming protein, permit glucose permeation across the outer membrane and inducible in nature. These results suggest that in the presence of aromatics, metabolism and transport of glucose in P. putida CSV86 is suppressed, thus allowing CSV86 to utilize aromatics preferentially.

Keywords: Preferential utilization, aromatic metabolism, glucose transport system, induction

References

Book of Abstracts III International Conference on Environmental, Industrial and Applied Microbiology (BioMicroWorld2009)
Lisbon (Portugal), 2-4 December 2009
Reactive Violet 12 dye decolorization by mycelial culture of *Trametes versicolor*
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The industry is a major source of pollution for water ecosystems. Industrial production of textile, cellulose and various chemicals is connected with synthetic dyes usage. The discharged effluents could have a hazardous influence on the environment. The biological treatment for synthetic dyes removal is a very perspective, environmentally protective and cost-effective way of solution for such problems. One of the recently used dyes is Reactive Violet 12. Its popularity is due to the effective cold dying properties. There is no any data in the scientific literature about the process of biodecolorization of wastewater obtained after treating with this dye.

The process of Reactive Violet 12 decolorization by *Trametes versicolor* strain 1 was investigated in this study. The experiments were carried out with different concentrations of dye (50mg/l and 125mg/l) and glucose (1, 2 and 3%) in a medium. The highest values of the specific decolorization rates (Q) of the processes carried out with different glucose concentrations were obtained in the presence of 1% glucose. The enzyme activity of laccase (IEC 1.10.3.2) was measured during the process of decolorization. A direct correlation between the observed enzyme activity and the effectiveness of investigated processes was proved. In spite of the fastest speed of decolorization registered in the medium complemented with 1% glucose the total decolorization was improved by increasing the initial glucose concentration in the medium. The higher concentrations of glucose maintained more percentage of decolorization due to the better growth of *Trametes versicolor*. Nevertheless it was established that maximal laccase activity was comparably equal in all described experiments (120 U/ml – 145 U/ml).

It was established that the best conditions for laccase production are in a medium containing 3% glucose. Correspondingly, the decolorization of 125mg/l Reactive Violet 12 dye in these conditions was 100 % completed in 360 hours.

Keywords: decolorization; laccase; Reactive Violet 12; *Trametes versicolor*

Removal of bentazon by liquid and solid state cultures of *Ganoderma lucidum*
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The herbicide bentazon is commonly used as a post-emergence herbicide in cereal crops. In Brazil, it is mainly used on peanuts, rice, beans, corn, soy-beans and wheat. Bentazon is degraded at a moderate rate by microorganisms in the soil environment. As consequence, after pesticide application, residues may remain in the crops, soil and natural water, constituting a health risk because of their toxicity. Removing bentazon from the environment in an ecologically responsible, safe, and cost-effective way is a top concern for land management agencies. Bioremediation using various microbial organisms is one way of doing it. In the last years, the capability of white rot fungi (WRF) to biodegrade several xenobiotics and recalcitrant pollutants has generated a considerable research interest in the area of industrial/environmental microbiology. WRF are the only microorganisms known to be able to degrade the highly recalcitrant natural polymer lignin because they possess a powerful enzymatic system formed mainly by peroxidases and laccases. *Ganoderma lucidum* is one of the most important and widely distributed WRF in the world and it is associated with the degradation of a wide variety of woods. The potential of *G. lucidum* and its enzymes in bioremediation processes is still far from being fully explored. Within this context, the objective of this work was to compare the removal of the herbicide bentazon by liquid and solid state cultures of *G. lucidum*. The fungus was cultured on potato dextrose agar Petri dishes (PDA) for up to 2 weeks at 28 °C. When the Petri dish was fully covered with mycelia, mycelial plugs measuring 10 mm in diameter were made and used as inoculum. *G. lucidum* was cultivated under liquid stationary or in solid state conditions at 28 °C in the dark. Three disks with 10 mm of diameter from the growing edge of the mycelium on PDA plates were transferred to 125 ml Erlenmeyer flasks containing 25 ml of mineral solution supplemented with corn cob powder at 1% as substrate (liquid cultivation) or to flasks containing 5 g of corn cob powder at 75% of moisture content (solid state cultivation). Different amounts of bentazon (0.50 m M) were added to the media. At periodic intervals 25 ml of cold water were added to the solid state cultures and the mixtures were shaken for 1 h at 4 °C. Liquid and solid state cultures were then filtered, and the filtrates were used as source of enzymes. For extracting the bentazon possibly sorbed on the fungal mycelia and residual corn cob, 25 ml of methanol were added to the insoluble materials obtained after aqueous extraction and the mixtures were shaken at 120 rpm in an orbital shaker for 2 h. To evaluate the residual bentazon, the combined aqueous and methanolic extracts were concentrated just to dryness by using a rotary evaporator. Each residue was reconstituted in 10 ml of a mixture of methanol/acetic acid 0.1 M (50:50). A HPLC system with a LC-20AT Shimadzu system controller, Shimadzu SPD-20 A UV-VIS detector, equipped with a reversed Shimpack C18 column (4.6 x 250 mm), maintained at 30 °C, was used for determining the residual amounts of bentazon. All samples in duplicate were filtered through a 0.22 μm filter unit before injection and the solvents were filtered through a 0.45 μm filter. The mobile phase was methanol:acetic acid 0.1 M (50:50) and the flow rate was 1 ml/min. Detection was done at 254 nm. The herbicide concentrations were determined using a calibration curve constructed with peak areas of authentic standards. Identification of bentazon in the samples was based on retention time (6.38 min) and fortification of the samples with standards. Laccase activity was determined with 2,2'-azino-di-(3-ethylbenzothiazolin-6-sulfonic acid) (ABTS) as the substrate. Oxidation of ABTS was monitored as absorbance increase at 420 nm. The Mn peroxidase activity was assayed by following the oxidation of MnSO4 in malonate buffer in the presence of H2O2. Manganese ions form a complex with malonate, which absorbs at 270 nm. One unit (U) of enzymatic activity was defined as the amount of enzyme required to produce 1 μmol product per min and was expressed as U/L. *G. lucidum* was able to grow in liquid and solid state cultures using corn cob as substrate. In control cultures, identical maximal laccase activities were 1,000 U/L in both types of cultivation. In relation to Mn peroxidase, solid state conditions allowed the obtainment of high Mn peroxidase activity (230 U/L), in comparison to that one obtained in liquid cultures (15.3 U/L). Bentazon had a negative effect on the mycelial growth (visual analysis) in both types of cultures. No growth was observed upon the addition of 25 and 60 mM bentazon in liquid and solid state cultures, respectively. In spite of the apparent growth inhibition, the herbicide enhanced the production of laccase to a maximal value of 1,800 U/L, using 2.5 mM and 30 mM bentazon in liquid and solid state cultures, respectively. The Mn peroxidase activity was only slightly improved by bentazon: using 10 mM of bentazon, the production of Mn peroxidase was 21 and 262 U/L in liquid and solid state cultures, respectively. After 10 days of cultivation, the residual bentazon present in the combined extracts was 47 and 12% of the initially added to liquid and solid state cultures, respectively. In the present work *G. lucidum* showed a considerable tolerance to bentazon when cultured on solid state conditions. The data suggest that under both types of cultivation, the fungus was able to degrade bentazon. Degradation, however, was more efficient under solid state conditions, where high levels of both laccase and Mn peroxidase activities were found. These observations suggest that both enzymes may have a role in bentazon degradation. These observations...
suggest the use of solid state cultures of *Ganoderma lucidum* in strategies designed to reduce the contamination of the environment by this herbicide.

**Keywords:** bentazon; *Ganoderma lucidum*; herbicides; laccase; ligninolytic enzymes; white rot fungus.

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**Removal of Heavy Metals in Wastewater Effluents in an Integrated Mode Using Supermacroporous Gels for Enrichment and Biogenic Sulphide for Subsequent Precipitation**

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Efficient wastewater treatment systems for the removal of heavy metals are needed. Available techniques suitable for capture of heavy metals (HM) prevailing at low concentrations are scarce and not as well developed. The capture of HM can be achieved by adsorbents. Enriched metal solutions can thereafter be precipitated by e.g. biogenic sulphide (BS). In this study, adsorbents based on supermacroporous gels (cryogels) and bearing metal-chelate functionalities (iminodiacetate residues (IDA) and ligand derived from derivatization of epoxy-cryogel with tris(2-aminoethyl)amine (TREN) followed by the treatment with bromacetic acid (defined as TBA ligand)) have been prepared and evaluated on capture with HM. The cryogels were prepared in plastic carriers, resulting in a desired mechanical stability. Sorption experiments for different metals (Cu$^{2+}$, Zn$^{2+}$, Ni$^{2+}$ and Cd$^{2+}$ with IDA adsorbent and Cu$^{2+}$ and Pb$^{2+}$ with TBA adsorbent) were carried out in batch and column modes. Obtained capacities with Cu$^{2+}$ were 74 and 19 mol/m$^3$ gel for TBA and IDA adsorbents respectively. The TBA ligand showed potentials for the removal of Cu$^{2+}$ ions from aqueous solutions at low concentration. About 80 and 90 % of Cu$^{2+}$ ions were captured with the TBA adsorbent after applying 20 L of Cu$^2+$ solutions (0.50 and 0.25 mg/L, respectively) in column mode. A suggested integrated treatment includes (1) capture of HM ions by the cryogel adsorbents followed by (2) precipitation of enriched HM using BS.

**Keywords:** Adsorption; Biogenic sulphide; Cryogels; Heavy metals; Water treatment.
Response of a denitrifying *Pseudomonas* to sodium benzoate

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Biodegradation of hydrocarbons by natural populations of denitrifying bacteria represents an effective means of elimination of petroleum contamination. These facultative microorganisms have an ideal mechanism of respiration using nitrate as the terminal electron acceptor in the absence of oxygen and can afford an extensive range of habitats. With different oxygen concentrations as compared to other microbial groups and thus form an ideal strategy for hydrocarbon removal.

An aerobic denitrifying bacterium was isolated from the sand dunes along the Arabian Sea and maintained on nitrate reduction medium (NRM) and denitrification medium (DM) with KNO₃ as the nitrate source. The culture was identified as *Pseudomonas aeruginosa* from its morphological and biochemical characterization and by 16s rRNA gene sequencing. When the culture was spot-inoculated in bromothymol blue medium with increasing concentrations of nitrate, it exhibited denitrification with up to 4% of nitrate concentration. Further, this investigation was directed at studying the response of the *Pseudomonas* culture to sodium benzoate (a model compound) and its effect on denitrification. The culture showed growth in a mineral salts medium with up to 3% sodium benzoate and exhibited the ortho mode of ring cleavage. The culture was inoculated in DM supplemented with 0.1% sodium benzoate and incubated under static conditions and on shaker conditions with 50rpm, 100rpm and 150rpm. Nitrite was formed and reduced within 6hr at 100rpm and within 8hr under static conditions, at 50rpm and at 150rpm as shown in Fig.1. The effect of different concentrations of sodium benzoate on nitrite formation and reduction was studied by growing the culture in DM supplemented with increasing concentrations of sodium benzoate. The supernatant was taken every 2hr and treated with sulphanilic acid and naphthaldehyde. The increase and decrease in nitrite was determined by measuring the absorbance at 540nm as illustrated in Fig.2. It was observed that with the inoculum from a benzoate medium, the nitrite was formed and reduced with 1% sodium benzoate after 8hr as compared to inoculum not exposed to benzoate which gave a reduction after 10hr (Fig.2).

The culture is a strong denitrifier and shows great potential in hydrocarbon bioremediation. Further work on the response of the culture to sodium benzoate with respect to growth, pigment production and emulsification activity will be presented.

**Keywords** hydrocarbon; denitrification; sodium benzoate; nitrite; *Pseudomonas*

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Screening of pentachlorophenol degradation ability of several fungi isolates from Tunisian soils

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All environmental compartments are linked together, but soil behaves as the main vector for pollutant migration as a receptor (e.g. agro-forestry additives, air pollution deposition), in transfer (e.g. migration to ground water, root-uptake) and decay (absorption, microbial transformation). It constitutes a diverse, invaluable and fragile resource. Soil degradation and pollution is increasingly affecting its sustainability and consequently its quality and normal function. Persistent organic pollutants (POPs) persist long in the environment may travel long distances from the source of application and are bio-accumulative in living organisms. In soil POPs strongly absorb to the humic matter, where microbial transformation by the endogenous soil microbe is a major component.

Man-made pentachlorophenol (PCP) is environmentally persistent (photo stable) and its water solubility together with its moderate mobility makes soil/water interaction acute, thus a good model for assessing the environmental decay of POPs.

Fungi are known for their diversity and remarkable ability to degrade complex and persistent natural materials such as lignin and chitin. In contrast to bacteria, fungi are able to extend the location of their biomass through hyphal growth. They are able to grow under environmentally stressed conditions such as low nutrient availability, low water activity and at low pH values where bacterial growth might be limited.

The study presented focuses on the capacity of fungal strains amongst the soil colonising communities to bioremediate PCP. The soil samples studied were systematically collected inside Tunisian Oak forests (summer/winter) and comprehensively analysed (chemical and microbial). This forest is at risk from PCP impact on the soil fungal colonising community.

The authors are deeply thankful to NATO sfp-981674 team members, especially to member of Tunisia team, lead by Prof. A. Hassen, who have executed the soil sampling. The work was partially supported by the projects: NATO dp sfp-981674.
Screening of Potential Biosurfactant Producing rhizospheric Microorganisms of fique (Furcraea sp) for potential soil bioremediation

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Aliphatic hydrocarbons are non-polar compounds, of low solubility in water, hydrophobic and with a high biocorrosion factor. It has been estimated that when these compounds are released into the environment they accumulate in soils, where due to their high affinity for organic C they are frequently found adhered to soil particles. Recently it has been suggested that certain rhizosphere inhabiting microorganisms from perennial plants such as fique, a native Agavaceae living in different ecosystems and that presents restoring effects on soils and is an ideal model for microorganism isolation with potential for soil bioremediation.

In this work, the production of biosurfactants was studied as well as their emulsifying ability of aliphatic hydrocarbons (kerosene) and the stability of the biosurfactants under extreme conditions. Thirty one Rhizobacteria (16 Pseudomonas, 9 sporedated Bacillus and 6 Actinomyces) were isolated from Fique roots coming from three distinct producing regions. 51.6% of the evaluated Rhizobacteria presented extracellular glycopolid production. From these biosurfactant producing isolates fifteen corresponded to the genus Pseudomonas and one to the genus Bacillus. Stable and compact emulsions of kerosene with the supernatant fluid of the culture were observed after 24 h of cultivation reaching maximal value of 69% at 120h of incubation. The emulsifying agent maintained its properties over a wide range of pH (2–9), at high salinity (20% NaCl), and during exposure to high temperatures (121 °C). The microbial bioemulsifier was effective at these extreme environmental conditions and was able to emulsify the tested pure aliphatic hydrocarbons.

The isolates with higher emulsification percentages and extracellular glycolipid production were identified using a Biolog® identification system as P. fluorescens, P. putida. The best production of 4.2 g/l was obtained when the cells were grown on minimal salt medium containing 2% (w/v) glycerol and 0.1% (w/v) ammonium sulfate 30 –C and 180rpm after 12 days. The optimum biosurfactant production pH value was found to be 7.0. The biosurfactant could reduce surface tension to 29mN/m and emulsified hexadecane up to E24±68.

The analysis of respiratory activity of microorganisms of active sludge cultivated on pre-radiated kerosene solutions showed that in case of KrCl- and XeBr-exilamp pre-radiation significant decrease of respiratory activity in the first cultivation days was not observed. Active sludge respiratory activity in 3-7-day period did not exceed the control. In case of active sludge cultivation with periodic mixing MCPA concentrations decreased 60%, and in 14 days decreased 87%. The analysis after 5 days revealed 2-methyl-4-chlorophenol in the media and after 14 this metabolite was not registered. After 5 days of active sludge cultivation KrCl- and XeBr- exilamp pre-radiated MCPA solutions revealed 4*10⁻⁶ M and 7*10⁻⁶ M concentrations of initial toxicant respectively. After 14 days MCPA and dehalogenated photolysis products were not registered. Moreover, 2-methyl-4-chlorophenol complete decomposition and decrease of chlorinated photo-product concentrations were observed in case of XeBr-exilamp pre-radiation.

Thus, UV-radiation of MCPA solutions allows not only speeding up MCPA utilization but also transformation of 2-methyl-4-chlorophenol, toxic to many microorganisms, into a compound less resistant to further biodegradation.

Sequential Photo-Biodegradation of MCPA with the Use of Exilamps
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Photolysis is one of perspective methods of degradation of organic compounds. Most experimental works on research of photochemical transformation of toxicants have been done with using of mercury lamps. However is known organic molecules effectively absorb UV-radiation with the certain of wavelength, that depends on spectral characteristics of the substance. It makes using exilamps as a source of UV-radiation very perspective to carry out more effectively phototransformation of organic compounds. The exilamps only start to be used in the scientific and applied purposes, including in the problems connected to preservation of the environment. Many aspects connected to their influence, still are not investigated at all.

The aim of present work was studying of efficiency of biodegradation, photolysis and sequential photo-biodegradation of MCPA.

It was shown that under UV-radiation of a KrCl-exilamp MCPA concentrations in water reduced from 2*10⁻⁴ to 2.2*10⁻⁵ M. Such herbicide concentration reduction was registered after 15-minute radiation and after that under radiation up to 60 minutes significant reduction of concentrations was not observed. Under UV-radiation of a XeBr-exilamp MCPA concentrations in water also reduced to 2.6*10⁻⁷ M, but further radiation caused herbicide concentration reduction to 10⁻⁷ M.

MCPA photolysis produced chloride ion and unbound chlorine concentration increase due to herbicide dehalogenation during photo-transformation. The chromatography mass spectrometry analysis showed that in spite of the length of a UV-radiation wave the first stage of MCPA photo-transformation was the formations of 2-methyl-4-chlorophenol. Radiation time increasing it was further transformed into 2-methylphenol, 2-methylhydroquinone and 2-methyl-4-chlorophenol. Stable and compact emulsions of kerosene with the supernatant fluid of the culture were observed after 24 h of cultivation reaching maximal value of 69% at 120h of incubation. The emulsifying agent maintained its properties over a wide range of pH (2–9), at high salinity (20% NaCl), and during exposure to high temperatures (121 °C). The microbial bioemulsifier was effective at these extreme environmental conditions and was able to emulsify the tested pure aliphatic hydrocarbons.

The isolates with higher emulsification percentages and extracellular glycolipid production were identified using a Biolog® identification system as P. fluorescens, P. putida. The best production of 4.2 g/l was obtained when the cells were grown on minimal salt medium containing 2% (w/v) glycerol and 0.1% (w/v) ammonium sulfate 30 –C and 180rpm after 12 days. The optimum biosurfactant production pH value was found to be 7.0. The biosurfactant could reduce surface tension to 29mN/m and emulsified hexadecane up to E24±68.

The analysis of respiratory activity of microorganisms of active sludge cultivated on pre-radiated /g582/g587/g586/g570 solutions showed that in case of KrCl-exilamp radiation CO₂ accumulation intensity was decreasing in the first three days, whereas later respiratory activity was increasing compared to clean active sludge and active sludge with a non-radiated herbicide.

In case of XeBr-exilamp radiation significant decrease of respiratory activity in the first cultivation days was not observed. Active sludge respiratory activity in 3-7-day period did not exceed the control. In case of active sludge cultivation with periodic mixing MCPA concentrations decreased 60%, and in 14 days decreased 87%. The analysis after 5 days revealed 2-methyl-4-chlorophenol in the media and after 14 this metabolite was not registered. After 5 days of active sludge cultivation KrCl- and XeBr- exilamp pre-radiated MCPA solutions revealed 4*10⁻⁶ M and 7*10⁻⁶ M concentrations of initial toxicant respectively. After 14 days MCPA and dehalogenated photolysis products were not registered. Moreover, 2-methyl-4-chlorophenol complete decomposition and decrease of chlorinated photo-product concentrations were observed in case of XeBr-exilamp pre-radiation.

Thus, UV-radiation of MCPA solutions allows not only speeding up MCPA utilization and but also transformation of 2-methyl-4-chlorophenol, toxic to many microorganisms, into a compound less resistant to further biodegradation.
Simultaneous decolorization and detoxification of Black Reactive 5 using TiO₂ deposited over glass

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During the dyeing process about 25% of the dye is not fixed and enters into the environment. Due to their complicated structure most of the dyes are resistant to biodegradation and the by products are more dangerous that the original dye. Heterogeneous photocatalysis whit TiO₂ offers an attractive advanced oxidation process for these pollutants. For this reason the Reactive Black 5 (RB5) dye was degraded by using UV-irradiated TiO₂ USP coated on glass. The treated effluent showed low toxicity with respect to the RB5 without treatment.

The TiO₂ was coated for sedimentation over glass and these films were dried and annealed at (450°C) for one hour twice. Later the films were introduced in a photoctalytic reactor of quartz (UV light 256 nm) with 120 ml of water and dye at 14 h. We used ascendants concentrations of dye (10, 50, 60, 70, 80 and 100 ppm), but the results shows that 70 ppm is the highest concentration that the system is able to degrader at 100% (0 ppm) in 14 h.

The sequencing batch experiments with TiO₂ were evaluated for 46 h and the results suggest that the films were able to decolorize 70 ppm of RB5 for 3 continuous cycles (100%).

Finally, a toxicity test with Daphnia magna revealed, that for killing half of the population in 48 h is necessary a minor concentration of BR5 to 70 ppm (CL₅₀: 25%) that of water after of 14 h of treatment with heterogeneous photocatalysis (CL₅₀: 60%).

These results suggest that UV-irradiated TiO₂ coated over glass may be considered as an adequate process for the discoloration and detoxification of the textile wastewater.

Soil microcosms for determination of growth by Streptomyces mirabilis P16B1 in heavy metal contaminated soil

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Streptomyces are a dominant group of soil bacteria which belong to the group of Actinobacteria. They are known for their complex life cycle, including mycelial growth and spore production, as well as their production of secondary metabolites, among them a large number of antibiotics. In comparison to pristine soils, heavy metal contaminated soils show much higher numbers of Gram positive bacteria, with bacilli and streptomycetes dominating over Gram negative proteobacteria or firmicutes. The former uranium mining site WISMUT in Eastern Thuringia, Germany, shows extreme environmental conditions, such as scant nutrients, intense salt load and low pH, followed by high metal content. The banks of the creek Gessenbach are characterized by exceedingly high concentrations of heavy metals as a consequence of the permanent inflow of acid mine drainage water over several decades of mining activity. Actinobacteria isolated from this hostile environment show high resistances against a range of heavy metals, including nickel, cobalt, cadmium or zinc. The extremely heavy metal resistant strain *Streptomyces mirabilis* P16B1 shows the ability to grow in highly contaminated soil from sample site K7 without the addition of any media ingredients even though there is a significantly higher content of a range of heavy metals, including mobile and adsorbed fraction metals as determined by sequential extraction methods. Scanning electron microscopy was used to detect the mycelium of the strain and spore-production on the surface and in the interior of the soil. It could be shown that inoculation with the strain has an effect of heavy metal availability in mobile and adsorbed fraction. The superoxide-dismutase-production of *S. mirabilis* P16B1 under natural conditions in microcosms was investigated by SOD-activity-assay and total protein-expression under artificial conditions with and without high nickel content by two dimensional gel-electrophoresis.

**Keywords**: Actinobacteria, Metal resistance, microcosm, SOD, protein expression,
Soluble Sulfate Removal from Effluent Water by Sulfate Reducing Bacterial Consortia

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Sulfate contamination in water causes various environmental and health hazards. High sulfate contamination has laxative effects on human, cause various skin problems and imparts an unpleasant taste to the water. Sulfate is released mainly as a byproduct of industrial activities like metal smelting, fuel gas scrubbing, and mining activities. The techniques for sulfate decontamination are many, like reverse osmosis, distillation, and ion exchange but they have many drawbacks when their efficiency is compared with the cost of technology. The alternative way of combating the problem is the exploitation of the technique of bioremediation. Areas like hot water springs, wetlands, and mining sites could be potential hotspots for isolation of Sulfate Reducing Bacteria. Sulfate Reducing Bacteria, commonly known as SRB are a heterogeneous group of microbes, which use sulfate as terminal electron acceptor. They use simple organic compounds like hydrogen, ethanol, methanol, lactate, propionate, pyruvate etc., as electron donors and reduce the sulfate to hydrogen sulfide as end product by dissimilatory sulfate reduction pathway. This hydrogen sulfide can react with metals and produce insoluble metal sulfides. The sulfate reducing bacteria are of wide technological interest because of their ability to reduce sulfate and form insoluble metal sulfide thus removing sulfate from waste water.

Here we have focused on (a) Isolation efficient SRB consortia from different parts of India and their characterization to understand their sulfate reduction efficiency (b) Technology I (c) sulfate contaminated effluent treatment and development of efficient SRB consortia.

Eight different SRB consortia were obtained from twenty two different samples screened. The consortia were grown in wide range of pH and temperature in order to understand sulfate reduction from different sulfate contaminated environmental water. Both turbidometric method and Ion-Exchange chromatography revealed that the most efficient consortia reduced a sulfate load of 2000ppm in 30 hours. The consortia can tolerate sulfate load of 7500ppm reducing the sulfate in 96hours. It can utilize lactic acid as a cheaper source of electron donor apart from sodium D-L lactate. Upon immobilizing the bacteria on corrugated sheet packed bed 10L bioreactor it reduced sulfate of 2000ppm in 120 hours where no anaerobiosis was generated externally and no temperature was maintained in order to reduce the energy consumption cost at pilot scale study. Molecular characterization of 182 clones from the 8 different consortia revealed that novel SRB along with different non-SRB. These sequences obtained have been submitted to the NCBI Genbank database under accession numbers EF069968 - EF069983, EF304455 - EF304467, GQ503570 - GQ503587 and GQ898863-GQ898881. The saturation curve clearly indicates that no further screening was needed to understand the population diversity. Shannon diversity index and Equitability Index speaks about the less diversity of the population which is due to the specific media used for cultivation specific SRB population.

Thus these microbial consortia with different efficiency of sulfate reduction would have great application in the treatment of soluble sulfate rich effluent water from different environmental sites. The diverse nature of the consortia like the wide pH and temperature tolerance with associated reduction would make them suitable for application at various sites. More over the immobilization of the consortia in 10L bioreactor without any external energy consumption would reduce the cost per se. But not limiting the study to this point, next step would be searching for a much cheaper source of substrate for reduction kinetics like using indigenous source of lactic acid instead of commercially available one. Bioaugmentation can also be done where the efficiency of the consortia could be increased either by adding some nutrients or manipulating certain conditions.

Keywords: Sulfate Reducing Bacteria, Bioremediation

Spent mushroom substrate from the industrial cultivation of P. ostreatus for discolouring complex chromo-baths for the textile industry: white rot fungi for a sustainable approach to wastewater treatment

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Synthetic dyes used in textile industry, are recalcitrant to degradation and toxic to different organisms. Physical-chemical treatments of textile wastewaters are not sustainable in terms of costs and produce large amounts of toxic wastes. Biological treatments can be more convenient and the lignin-degrading extracellular enzymatic battery of basidiomycetes have received extensive attention for their capacity to discolour synthetic dyes. Many basidiomycetes are edible mushrooms whose industrial production generates significant amount of spent mushroom substrate (SMS) with residual high levels of lignin-degrading extracellular enzymatic activities. We have demonstrated that the SMS deriving from the cultivation of the basidiomycetes Pleurotus ostreatus (white rot fungus) is able to discolour anthraquinonic, diazo and monoazo dyes when incubated in dying chromo-reactive and chromo-acid baths. The capacity of the SMS has been tested on industrial chromo-baths, instead of aqueous solution of single dyes. In fact, the former carries a higher similarity to the real industrial wastewaters, since containing auxiliaries of the dying process (mainly surfactants and salts), that actually are also released in wastewaters and can interfere with the discoloring process. Moreover, the chromo-baths here tested for discoloration contained chemicals at concentrations definitely higher than the one recovered in the real wastewaters: dyes and auxiliaries in concentration up to the 20% of the initial chromo-bath mass used in the dying process. It is estimated that the amount of textile dyes released in wastewaters account for the 10% of the total used in the industrial process. A total of 70-90% of discoloration occurred in 24 hrs for all the chromo-bath tested. Laccase was the lignin-degrading extracellular enzyme involved in the discoloring process. The exploitation of the low cost SMS in the treatment of textile was reinforced by a toxicological test on a human epithelial cell line (WISH) and the estimation of the germination index (GI%) of Lactuca sativa, Cucumis sativus, has been performed, showing the loss of toxicity of the chromo-baths after being discoloured by the SMS.

Keywords: anthraquinonic dye; diazo dye; laccase; monoazo dye; white rot fungi; spent mushroom substrate
Stimulation of polycyclic aromatic hydrocarbon biodegradation by nitrate and sulfate amendment to sediment along a natural salinity gradient

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Although it has been established that polycyclic aromatic hydrocarbons (PAHs) are biodegradable under anaerobic conditions, it is not clear how widespread this activity is and whether primarily laboratory-based experimental results from marine and estuarine sediments can be extended to the field. Surficial and depth-resolved sediment with a range of polycyclic aromatic hydrocarbon (PAH) pollution was collected from field sites spanning a natural salinity gradient from ~20 ppt salinity (near marine) to fully freshwater. Sediment samples were transported to the laboratory for preparation of laboratory and field microcosm incubations. Sediment for laboratory experiments were amended with nitrate or sulfate reducing electron acceptors, as well as phenanthrene and naphthalene as bioavailable PAHs to assay the broad scale effectiveness of biostimulating anaerobic respiring bacteria as a remedial strategy for PAHs. Sediment microcosms were prepared by amending the sediment with nitrate or sulfate and packing them in multiple field microcosms (one for each sample time and incubation condition) consisting of polycarbonate tubes with sealed bottoms with the top open and flush with the sediment water interface. The microcosms were placed back in the field and incubated over extended periods with destructive sampling by removal of an entire core at sampling time intervals. Over time, sulfate stimulated biodegradation of both phenanthrene and naphthalene in nearly all sites in the laboratory study. In contrast, high levels of nitrate did not stimulate naphthalene biodegradation in some sites. In contrast, most native PAHs with more than 3 rings were not removed significantly in field experiments, which is likely due to the lower levels of PAH pollution in the test sediments than in the PAH-amended laboratory incubations. Electron acceptor utilization kinetic studies were performed to determine whether amended anaerobic electron acceptors would be rapidly utilized for non-targeted utilization (i.e. by sediment organic matter oxidation via denitrification or sulfate reduction) under different temperatures representing spring/fall, summer and winter temperatures; as well as in the field incubations. Results demonstrate that electron acceptor utilization rates were significantly lower in the sulfate stimulated sediments than those observed in the nitrate stimulated sediments, indicating that more sulfate may be available specifically for PAH biodegradation. Further, Arenhius plots provide activation energies for these activities consistent with those observed in methanogenic conditions in contaminated sediments. These results suggest that sulfate may be a more widely applicable anaerobic electron acceptor for stimulating anaerobic PAH biodegradation in sediments from a variety of salinities. Further, PAH bioavailability is likely a key limiter in contaminated sediments due to the non-targeted oxidation of sediment organic matter and resultant utilization of amended electron acceptor.

Keywords: polycyclic aromatic hydrocarbon; PAH; sulfate reducing bacteria; denitrification; bioremediation; sediment

Sustainable remediation of polycyclic aromatic hydrocarbon contaminated soils using a two step bioremediation process.

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Concern about the impact of polycyclic aromatic hydrocarbons (PAHs) on human and environmental health has resulted in the listing of sixteen PAHs as priority pollutants on the Environmental Protection Agency’s contaminants list. High molecular weight PAHs are highly recalcitrant contaminants, and therefore effective remediation strategies must be capable of releasing and degrading PAHs in soils to produce readily biodegradable, partially oxidised metabolites with increased bioavailability compared with the parent compound. Various biotic and abiotic remediation techniques have been applied to PAH contaminated soils, including incineration, in-situ washing and Fenton’s oxidation. However all of these techniques effectively destroy soil biological function. Bioremediation offers the potential to safely remediate the soil; however the bioremediation of PAH contaminated soil is difficult and time-consuming using current methodologies.

In this study the treatment of polycyclic aromatic hydrocarbon contaminated soil using Fenton’s oxidation followed by a 6 week bioremediation field trial involving additions of compost or poultry manure was investigated. The soil was sampled from a disused gas manufacturing facility located in South Australia. The original concentration of PAH was 207 mg kg\(^{-1}\) with the benzopyrene concentration 13 mg kg\(^{-1}\). The effect of Fenton’s oxidation and subsequent compost or manure amendment on PAH concentration and on soil physical and biological parameters including soil respiration rate was assessed.

Following Fenton’s oxidation of soil using 1% (v/w) addition of 10% H\(_2\)O\(_2\), no significant reduction in PAH was detected. However 6 weeks following the addition of either spent mushroom compost or poultry manure to the soil immediately after Fenton’s oxidation a reduction in PAH concentration of 63% was observed (77 mg kg\(^{-1}\)), with benzopyrene concentration being reduced by 69% (≤5 mg kg\(^{-1}\)). These levels fall below the Health Investigation Level for factory and industrial sites according to Schedule B (1)-EPA Guidelines on Investigation Levels for Soils and Groundwater. In the absence of Fenton’s oxidation treatment, the addition of compost or manure resulted in a soil PAH level of 130 mg kg\(^{-1}\) and a benzopyrene concentration of 8 mg kg\(^{-1}\) after 6 weeks incubation.

In terms of biological activity, soil respiratory activity, after 6 weeks incubation was greatest in the Fenton’s treated soils amended with compost or manure (76 and 113 mg CO\(_2\) m\(^2\) h\(^{-1}\) respectively). Untreated soils and unamended Fenton’s treated soils showed significantly lower levels of soil respiratory activity (approximately 12 mg CO\(_2\) m\(^2\) h\(^{-1}\) respectively), confirming the restoration of biological activity in these treatments.

The results obtained suggest that Fenton’s oxidation of PAH contaminated soils followed by amendment with mushroom compost or poultry manure provides increased reduction of PAH contaminant concentration to below soil investigation level F suggesting the potential of this sustainable remediation technology. This is possibly due to enhanced microbial degradation of desorbed PAHs produced during the oxidation process when compost or manure amendments are present, and also due to increased total microbial numbers, and improved soil physical and chemical properties.

Keywords: bioremediation, Fenton’s oxidation, polycyclic aromatic hydrocarbon contaminated soils, sustainable remediation
Synthetic dye degradation by complex pellets of white-rot fungus *Trametes versicolor*

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Effluents containing textile dyes are usually discharged in large quantities worldwide into natural water bodies. The presence of these compounds can cause problems due to the possible entrance into the food chains of humans and animals. Once in the environment, they can show their toxic and genotoxic effects on organisms. Therefore, it is necessary to remove dyes before effluent discharge. Different treatments can be used for dyes removal; however, some of them can cause toxicity to the microorganisms of the biological system.

In recent years several studies have been demonstrated that white-rot fungi are able to decolorize and remove a wide variety of structurally diverse pollutants including synthetic dyes. The fungi present the advantage over other microorganisms due to their extracellular ligninolytic enzyme systems composed by manganese peroxidase (MnP), manganese-independent peroxidase (MIp), lignin peroxidase (Lip) and laccase (Lac). Therefore, the main objective of this study was to evaluate the synthetic dye degradation by pellets of white rot fungus *Trametes versicolor*.

Complex pellets of white-rot fungus *Trametes versicolor* was formulated with mycelium, activated carbon and sawdust (2:1:1) for the synthetic dye degradation. The pellets were formed by a center of activated carbon and sawdust surrounded by fungal mycelium (Figure 1).

The degradation was determined in modified Kirk liquid medium with an initial concentration of each dye of 100 mg L⁻¹ of Reactive Black 5 (RBS5), Acid Orange 6 (AO6), Reactive Orange 16 (RO16), Basic Violet 4 (BV4) and Blue Remazol Brilliant (BRB). The flasks were incubated at 25 °C, under agitation at 100 rpm, by 15 days. During the incubation period color degradation and ligninolytic enzyme activity (lignin and manganese peroxidase, laccase and manganese-independent peroxidase) were evaluated.

The results obtained showed that the dyes degradation by complex pellets of *Trametes versicolor* was: 93, 82, 95, 16, 75 and 95% for Reactive Black 5, Acid Orange 6, Reactive Orange 16, Basic Violet 4 and Blue Remazol Brilliant respectively. Manganese-dependent peroxidase was 30 U L⁻¹ and less activity was obtained with lignin peroxidase (1,4 U L⁻¹).

Figure 1. a) Degradation of AO6, b) Degradation of BRB, c) Control and degradation of RBS5 d) Pellet morphology

Figure 1 shows the degradation of acid orange 6 (a), blue remazol brilliant (b), control and reactive black 5 (c) and the *A. discolor* pellets morphology. The faster degradation was for the RBS and BRB dyes. In conclusion, the application of complex pellets of *Trametes versicolor* can be potentially used for the biodegradation of synthetic dyes.

Acknowledgments: Financed by FONDECYT 1090678 Project and partially by FONDECYT 3080803.

Keywords *Trametes versicolor*, complex pellet, synthetic dye, ligninolytic enzymes

Taxonomically distinct ETBE-degrading communities, originated from the same site, are dominated by novel *Mesorhizobium* and *Hydrogenophaga* species

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Ethyl tert-butyl ether (ETBE) is a gasoline additive widely used in Europe. The contamination of aquifers by ETBE during accidental petroleum spills is a serious problem since ETBE is an extremely soluble, recalçitrant compound. So far, only few aerobic bacterial isolates were shown to degrade ETBE and the biodegradation possibly involves ETBE oxidation by cytochrome P-450 (EthB). Though important for bioremediation purposes, the information on the potential of natural communities to degrade ETBE is scarce. The aim of this study was to characterize bacterial communities from a gasoline-contaminated aquifer in terms of (i) their capacity to degrade ETBE, (ii) taxonomical composition, and (iii) presence and diversity of the ethB gene.

Groundwater samples from a contaminated site in France, taken from a plume (P) under a petrol station (ETBE being the main pollutant at concentration ~ 200 mg.l⁻¹) and upstream the plume (control, C), were used to establish a microcosm experiment. Triplicate microcosms were cultivated aerobically with ETBE as a sole source of carbon and the rates of ETBE degradation were recorded over a year. The taxonomical composition of bacterial communities from the aquifer and the microcosms was assessed with 16S rRNA microarray and cloning/sequencing. The ethB gene was assessed by a specific PCR and cloning/sequencing.

The aquifer bacterial communities differed markedly between the plume (P) and the upstream control sample (C). The ETBE-degradation gene ethB was detected in the P but not in the C water sample. In spite of the different initial conditions, both the P- and C-microcosms degraded ETBE, and ethB could be detected afterwards in both of them. However, the taxonomical composition of microcosm communities did not convert and differed also from the initial aquifer communities. The C-microcosm became strongly dominated by *Mesorhizobium* spp. and the P-microcosm by *Hydrogenophaga* phylotypes. None of the previously described ETBE-degrading species was found within the microcosms, indicating that they harbor novel ETBE-degraders. In conclusion, ETBE degradation may be accomplished by taxonomically different consortia selected under the same conditions (presence of ETBE, aerobic cultivation) from distinct aquifer communities, possibly involving horizontal gene transfer.

Keywords Aquifer, bacterial community, biodegradation, bioremediation, ethyl tert butyl ether (ETBE), pollution.
The remarkable adaptability of *Rhodococcus erythropolis* cells

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*R. erythropolis* cells present a considerable natural tolerance and adaptation ability to compounds usually toxic to other bacterial strains, including terpenes (1), hydrocarbons (2) and aromatic compounds such as toluene and xylene (3). The adaptation mechanisms described in *R. erythropolis* involve: i) alterations at the cell wall and membrane composition; ii) modifications of the physicochemical properties of the cell surface; iii) degradation or bioconversion of the toxic compounds; iv) cell aggregation, and v) production of exopolymers substances.

The tolerance and ability to adapt to organic solvents can be valuable in biocatalytic and bioremediation processes. The cells are able to change their hydrophobicity and surface charge in response to the carbon source when grown in C6-C16 n-alkanes which are good solvents for biocatalytic processes (3). Since these cells also present sites where hydrocarbons can adsorb on the surface or in the inner part of the cells envelope (4), the cells become very hydrophobic. In organic:aqueous phase systems, the cells migrate rapidly towards the organic phase and the increased hydrophobicity helps the cells to adhere directly to the organic phase, thus overcoming the low bioavailability of compounds with low water solubility (5). This could be used to increase the production of carvone to 1.0M at a high productivity and yield, thus overcoming the inhibition usually observed at a concentration of carvone of only 25 mM. The adaptation was achieved by exposing the cells to slowly increasing concentrations of both substrate and product, in the presence of the solvent, prior to the biotransformation (3).

A similar strategy, during which the cells were adapted by doubling the concentration of toluene when the cells had finish the degradation of a certain amount, allowed the cells to metabolise concentrations up to 4.9M toluene in n-dodecane (3). The cells adapted to high concentrations of toluene also showed a substantially increased resistance to 50% ethanol and to concentrations of Betadine® (a strong broad-spectrum topical biocide) and Micropur® tablets (used to sterilize water) that usually kill all bacteria, indicating cross-resistance mechanisms between different compounds. A significant increase in *R. erythropolis* resistance to antibiotics was also observed after adapting the cells to antineoplastic agents.

*R. erythropolis* cells adapt the composition in fatty acids of the cellular membrane in response to the carbon source or to the presence of xenobiotics, and can even change the cell surface charge to positive values when grown on n-tetradecane and n-hexadecane. They can respond to high concentrations of recalcitrant compounds such as toluene and xylene by degrading the compounds or converting them to non-toxic forms. *R. erythropolis* can also, under stressful conditions, aggregate and produce exopolymers substances to protect the cells. These substances can act as biosurfactants, being able to decrease the surface tension of the medium up to 23 mN/m, indicating that they are among the most powerful natural biosurfactants known. Since these cells present a broad array of enzymes with potential for the production of commercially interesting compounds and for the metabolism of recalcitrant organic compounds, the adaptability of the cells can further broaden their application in biocatalysis and bioremediation processes.

**Keywords** bacterial adaptation; biocatalysis; bioremediation

**References**


Tolerance and stress response of the saprobe macrofungi *Macrolepiota procera* to nickel

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Nickel (Ni) is an essential element for many organisms however it is very toxic at high concentrations and also depending on the species. In macrofungi the mechanisms underlying their Ni-tolerance are poorly documented. We examined, for the first time, the participation of the antioxidative system in the *Macrolepiota procera* exposed to different Ni²⁺ concentrations and their relation with Ni-tolerance. The effect of the pH on Ni-tolerance was also evaluated. The fungus was cultivated on solid medium with different Ni concentrations (0.05, 0.2, 0.8 mM) at pH 4, 6 and 8, and fungi growth and Ni uptake was determined. The antioxidative enzymes, catalase (CAT) and superoxide dismutase (SOD) and the production of hydrogen peroxide (H₂O₂) were evaluated on fungal submerged cultures within first hours of Ni exposure. Results showed that *M. procera* growth decreased when Ni concentrations increased, reaching a maximum growth inhibition (higher than 80%) up to 0.2 mM of Ni. The Ni uptake increased proportionally to Ni increase in the medium. Both Ni-tolerance and Ni-accumulation were affected by media pH. Microscope observations showed differences on the size of spores produced by fungi at different Ni concentration. Ni exposure induced oxidative stress, as indicated by the production of H₂O₂, which levels seem to be regulated by the antioxidant enzymes SOD and CAT. The time variation pattern of SOD and CAT activities indicated that the first has a greater role on the alleviating stress. The results obtained suggested that the *M. procera* tolerance to Ni is associated with the ability of this macrofungi to initiate an efficient antioxidant defense system.

**Keywords**: *Macrolepiota procera*; Nickel; stress; Ni-tolerance; Ni-accumulation; hydrogen peroxide; antioxidant enzymes

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**Thermophilic Bacteria Degrading Poly(Vinyl Alcohol)**

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PVA is water-soluble and recalcitrant against biodegradation to eventually contaminate the water system.

PVA degradation mechanism was composed of two step reaction in sequence, i.e., the oxidation of the hydroxyl groups and the cleavage of the C-C bonds.

A lot of effort has been devoted to isolating PVA degrading microbes to enhance the effectiveness of the PVA-containing waste water treatment facilities. PVA degrading microbes are not ubiquitous at all but are localized in certain area.

In this study, the activated sludge harvested from the PVA producing factory was subjected to the enrichment culture in the mineral minimum medium containing PVA as a sole source of carbon. A portion of the enrichment culture was diluted with sterile saline and spread on the PVA-MM medium at 55°C for 10 days. Preliminary selection of the PVA degrading strains was carried out based on the growth rate in the mineral medium, and final selection was performed by measuring the size of the clear halo zone around the colonies.

The preliminary selection yielded 13 purely isolated strains and total 3 strains were chosen among the 13 strains after the final selection. Two strains out of the 3 strains, HB12 and HB13, did not form the clear halo in the respective pure culture at 55°C. However, when the two strains were inoculated in pair, the clear halo was formed at the same temperature. In contrast, the remaining one strain out of the 3 strains formed the clear halo even in the axenic culture. These results indicate that the two former strains constitute symbiotic pair, while the latter one strain can degrade PVA for itself without indebtedness to other strains. The halo zone size formed by HB1 and by HB12+HB13 pair was both larger than that formed by SB68+SB69 pair and by *Achromobacter cholinophagun*. In contrast to the 3 former thermophilic microbes, the latter 3 strains were mesophilic bacteria (Lee and Kim 2003).

Enhancement of the PVA degradation activity was attempted by performing acclimation of the isolated strains through 5–6 times of repeated culture in the mineral medium containing 0.1% of PVA as a sole source of carbon. The agar diffusion test results confirmed that the acclimation raised the PVA degradation activity considerably for both HB1 single strain and HB12+HB13 symbiotic pair.

Identification of the isolated strains was carried out according to Micro Station System (Biolog. Inc. USA). The single strain, HB1, which degraded PVA for itself without any externally added growth cofactor, was identified to be *Geobacillus tepidamans*. The phylogenetic tree of the strain, HB1, was constructed based on 16S rRNA sequence analysis. However, the genera HB12 and HB13 could not be identified clearly.

The HB12+HB13 pair formed larger halo than *G. tepidamans* single strain at 55°C. The size of the clear halo formed by HB12+HB13 pair decreased more precipitously than that formed by the single strain as the temperature rose to 60°C. And finally HB12+HB13 pair did not form the clear halo at 65°C, while the single strain still built up the clear halo 14 mm in diameter, indicating that the single strain was more thermally stable than the symbiotic pair. However neither the single strain nor the symbiotic pair could not form the clear halo when the temperature rose to 70°C.

After 20 days of the biodegradation, 93% of PVA was mineralized into CO₂ by *G. tepidamans* when the PVA content was 0.01%. Comparing the biodegradability of PVA achieved by *A. cholinophagun* at 28°C, which was about 40% during the same period of time (Lee and Kim(2003)), it can be said that the isolated single strain is a potent PVA degrader.

The biodegradability of PVA was reduced slightly when 0.01% of the basic dye (Black REH; Seil, Korea) was incorporated, while the biodegradability was significantly reduced when the reactive dye (Yellow 54G, S-025; Sunfix Supra Brill), the acid dye (N/Blue R; Unicet), or the dispersive dye (Blue SD; Terasil) was added at the same concentration. When the symbiotic pair HB12+HB13 instead of *G. tepidamans* single strain were inoculated into the medium, the biodegradability was increased slightly due to the basic dye incorporation. However, addition of the reactive dye, the acid dye, or the dispersive dye considerably depressed the PVA biodegradation activity of the symbiotic pair HB12+HB13 without exception.

**Keywords** Poly(vinyl alcohol); Biodegradation; Thermophilic bacteria; *Geobacillus tepidamans*

Uranium adsorption by *Articulospora tetracladia*: can aquatic hyphomycetes be natural bioremediators of uranium contaminated streams?

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Uranium is an important environmental contaminator in some areas of the world, including Portugal, where there are several abandoned uranium mines. At these locations, the uranium concentration in the water can be as high as 1.8 mg/L, which can potentially affect the aquatic biota. The effect of uranium has been addressed on several organisms, and microorganisms have also been studied for their ability to adsorb and accumulate uranium. However, no such studies have been conducted on aquatic hyphomycetes (freshwater fungi), which are major players on the decomposition of submerged organic matter, a key ecosystem level process in shaded streams.

The kinetics of uranium adsorption by *A. tetracladia* mycelium was relatively fast with 18% (200 μg/L) – 50% (2000 μg/L) uranium remaining in solution after 15 minutes. The maximum uranium uptake observed in this study (~140 mgU/gDM at 2000 μg/L) was higher than most reported in the literature. The fitting of the uranium uptake data to the Freundlich isotherm indicates monolayer uranium adsorption at the surface of the mycelium. The stability of the uranium monolayer is high (n=1), as well as the adsorption capacity given that at 1 μg/L, the uranium uptake is 1.73 mgU/gDM. Since the uranium uptake was not significantly different between live and dead mycelium, the uranium adsorption over the 6h study period probably resulted from a biodegradable process, independent of biological activity. The applicability of the Michaelis-Menten-type model indicates that adsorption at the mycelium surface progresses towards saturation, indicating that the limiting factor for uranium binding is the number of surface sites; maximum uranium uptake rate was 182 mgU/gDM, and 196 μg/L was the half saturating uranium concentration. The distribution coefficient values found for *A. tetracladia* were among the highest reported (Kd=4820 mL/g), which indicates its ability to accumulate uranium from very dilute solutions (stream water). The extraction factors varied between 28 and 41, which confirms that mycelium of *A. tetracladia* can be considered a good biosorbent. Aquatic hyphomycetes seem to have the potential to act as natural bioremediators of streams running through uranium contaminated areas. However, given that fungal mycelia constitute food for aquatic invertebrates, the accumulation of uranium might scale up to higher trophic levels.

**Keywords**: adsorption; aquatic hyphomycetes; *Articulospora tetracladia*; uranium contamination

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A method for detection of *Rhizomucor miehei* lipase activity

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*Shizomucor miehei* lipase (triacylglycerol acylhydrolase; EC 3.1.1.3) is an enzyme that catalyzes the hydrolysis of triacylglycerols to diacylglycerols, monoacylglycerols, fatty acids and glycerol, at the interface between aqueous and the lipid phase [1]. This lipase is region-specific, catalysing reactions at 1,3 positions on the triacylglycerols. Thus, the enzyme is a potential biocatalyst for the synthesis of lipids and the production of fatty acids, which is an increased demand for new sources of lipases. In this work, we report a method for detection of lipase activity in a *Rhizomucor miehei* grown on solid media [2].

Solid media for lipase activity assay was prepared by adding an emulsified solution of 40% (w/w) olive oil, 10% (w/w) Tween 80 and 50% (w/w) distilled water to medium containing 2% (w/w) agar in water. Water agar plates containing a final concentration of 0.05% (w/w) emulsified olive oil, were used for detection of lipase activity. Mycelium from *R. miehei* was grown on Malt extract agar, heat to boiling with frequent agitation and sterilise by autoclaving at 115 ºC for 15 minutes. Liquid media were tested at different temperatures, agitations, adding olive oil and 0.1M Tris-Hcl, pH=7.8. Then, the mycelium extract was placed on the agar surface and incubated at 28 ºC during 3 days.

Lipase activity was detected as a clear crown on agar surface around the mycelium extract. Clear crown was the result of the olive oil degradation by the fungal lipase. Thus, the conditions used to grow *R. miehei* were the optimal to produce the enzyme. In conclusion, we report a method to efficiently detect the lipase activity from *R. miehei*. This method could be used to check lipase activity in other fungal species.

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**Keywords**: lipase, *Rhizomucor miehei*, lipids.
A new lipase-catalyzed biodiesel by response surface methodology using *Rhizopus oryzae* derived lipase

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**Introduction.** Biodiesel has recently become more attractive because of its environmental benefits and the fact that it is made from renewable resources. Currently, biodiesel is commercially made by alkali-catalyzed transesterification of an oil or fat with an alcohol, usually methanol, a process that shifts the glyceride fatty acids from glycerol to methanol, producing fatty acid methyl esters and glycerol [1]. Though efficient in terms of reaction time, the chemical approach to synthesize biodiesels from triglycerides has drawbacks, such as the difficulty in the recovery of glycerol and the energy-intensive costs of the process. In contrast, biocatalysts allow for synthesis of specific alky esters, easy recovery of glycerol or no production, and transesterification of glycercides with high free fatty acid content [2]. Therefore, the production of enzymatic biodiesels by lipase-catalyzed chemical reactions under mild conditions has become of commercial interest. An optimized enzymatic synthesis of biodiesel improves the conversion yield and reduces the cost of production in most favourable conditions. The present work focuses on the reaction parameters that affect lipase from *Rhizopus oryzae* (LR) catalyzing the transesterification of sunflower oil with ethanol in free solvent media. The main objectives of this work were to develop an approach that would enable us to better understand relationship between the variables (reaction time, temperature, pH, enzyme amount, substrate molar ratio, and added water content) and the response (percent weight conversion) and to obtain the optimum conditions for biodiesel synthesis using multilevel factorial design.

**Results and discussion.** Results have been summarised in Figure 1. You can see the great influence of the ratio oil / ethanol (proportion), and to lesser extent of pH, while other parameters did not affect significantly.

**Conclusions.** Lipase from *Rhizopus oryzae* can be used as biocatalyst at optimum conditions to prepare a new kind of biofuel with composition and properties suitable to use in diesel engines. This method has the advantages of avoiding the generation of glycerol as byproduct in the process and its short reaction time. This process minimizes waste generation and maximizing efficiency of the process.

**Acknowledgements.** This research has been supported by the Instituto Andaluz de Biotecnología (Junta de Andalucía, Project BIOANDALUS 08/13(L35), Consejería de Educación y Ciencia de la Junta de Andalucía (FQM 0162), (FQM 0191), Ministerio de Educación y Ciencia Project CTQ 2007-65754-PPQ, Ministerio de Ciencia e Innovación CTQ 2008-01330(BQ)).

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**Keywords:** Lipase; *Rhizopus oryzae*; Biofuel; Ethanolysis; Fatty Acid Ethyl Ester (FAEE); Monoglyceride

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**Bacillus popilliae: Detection and Biochemical Characterization of Pectinolytic Activity**

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**Introduction.** Pectin is a complex set of polysaccharides that are present in the middle lamella between plant cells where it helps to bind cells together. Pectin degradation involves a complex combination of pectinolytic enzymes, two groups can be observed: pectin esterases which remove methoxy groups and the depolymerizing enzymes (hydrolyases and lyases) which degrade the glycosidic bonds along the polymer backbone. Pectate lyase breaks down polygalacturonic acid (PGA) through a transelimination mechanism. This enzyme is one of the most crucial enzymes in the depolymerization of pectin. (1). Pectate lyase are widely distributed among microbial plant pathogens (2, 3). They have also been found in saprophytic micro-organisms, including the genus *Bacillus* (3). Pectinases are enzymes of industrial interest they are widely used for juice and wine clarification. Alkalophilic pectinases have found immense use in the degumming of ramie fibers. Today the study of pectinases from microbial systems has caused large interest, since they have been found to be effective for scouring of crude cotton fiber (4). So far, there is no report on the pectinolytic activity from *B. popilliae*. The aim of this work is to get knowledge on the pectinolytic activity detected in the culture supernatant of *B. popilliae*.

**Materials and Methods.** *B. popilliae* was obtained from CINVESTAV, México. Bacteria were cultivated (30°C, 24 h, 200 rpm) in nutritive broth, supplemented with 0.1% glucose, pectin or galacturonic as the only carbon source (5). Cells were removed and the culture supernatant was used as a source of pectinase activity. Lyase activity was achieved valuating unsaturated products creation from polygalacturonic acid (pectin lyase activity) at 235 nm by spectrophotonic assay, as described (6). The reaction mixture contained 1.5 ml of 0.1% of substrate into a buffer solution with the convenient pH. Incubation last 1 min at 55°C and then 1.5 ml of water was added. One unit of activity was defined as the necessary amount of enzyme to promote an increment of one unit in absorbance.

**Results and discussion.** Pectinase activity was detected in the culture supernatant from *B. popilliae* grown on nutritive broth supplemented with 0.1% glucose, pectin or galacturonic as the only carbon source. The highest and the lowest enzyme activity were observed when pectin and glucose were used as substrate, respectively (Fig. 1). Two maximum of lyase activity were observed when *B. popilliae* was grown on nutritive broth supplemented with 0.1% glucose, pectin or galacturonic as the only carbon source. The highest and the lowest enzyme activity were observed when pectin and glucose were used as substrate, respectively (Fig. 1). Two maximum of lyase activity were observed when *B. popilliae* was grown on pectin, at pH values of 9 and 11 (data not shown).

Further studies, using metal ions (Ca, Co, Mg, Mn and Zn) (Fig 2A) and quelating agents like EGTA, strongly suggest that *B. popilliae* presence of Ca but deeply inhibited in presence of EGTA, even when Ca was present (Fig. 2B).

**Fig. 1 Pectinolytic activity of *B. popilliae* using different carbon sources in the cultivation media.**

**Fig. 2. A Studies using different metal ions. B Effect of ions Ca and quelating agents like EGTA in the pectinolitic activity and**

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Of the original text.
Conclusions.

- *B. popilliae* presents two maximum of pectinolytic activity, at pH values of 8 and 11. The pectinolytic activity from *B. popilliae* is inducible and the best inductor is pectin; while this activity was negatively affected by glucose as carbon source.

- *B. popilliae* produce and secrete at least two alkaline pectinases. The requirement of Ca suggest that one of pectinolytic enzyme is a pectate lyase.

Keywords: *B. popilliae*, polygalacturonic, pectin, lyases

References

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Bacillus subtilis CwIP in the SP-beta prophage comprises two novel cell wall hydrolase domains

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Precise cell wall digestion by hydrolases is a very important event for not only bacteria but also bacteriophages. We reported that the *cwlT* gene in *B. subtilis* is located in the conjugative element (ICE*Bs1*) (1) and the gene product, CwlT, comprises two cell wall hydrolase domains. The C-terminal domain defined as a NlpC/P60 exhibits d,l-endopeptidase activity similar to those of LytF, CwlS and CwlO (2-4); however, interestingly the N-terminal domain exhibits only muramidase activity though this domain was previously described as a soluble lytic transglycosidase (SLT). CwlT degraded cell wall very efficiently.

In this conference, we present characterization of the product of *yomI* (renamed CwIP [cell wall lytic enzyme related with phage]) which is located in the SP-beta prophage region. CwIP also has SLT domain, which is similar to that of *E. coli* Slt70 (characterized as a lytic transglycosylase). Additionally, CwIP has peptidase M23 domain, which is similar to that of *Staphylococcus aureus* LytM (Gly-Gly endopeptidase). The SLT domain of CwIP exhibits hydrolytic activity toward *B. subtilis* cell wall, however, the products of the enzyme does not include anhydro-N-acetylmuramic acid (anhMurNAc), as found on reverse-phase (RP)-HPLC, mass spectrometry (MS), and MS/MS analyses, indicating CwIP has only muramidase activity. On the other hand, the peptidase M23 domain of CwIP showed hydrolytic activity and cleaved the cross-linkage of d-Ala-diaminopimelic acid as a d,d-endopeptidase, as found on RP-HPLC. Interestingly, the M23 domain of CwIP is a quite unique enzyme as a Zn²⁺-independent endopeptidase, though all characterized M23 peptidases and enzymes similar to CwIP depend on Zn²⁺. Moreover, both the two domains of CwIP could hydrolyse only *B. subtilis* cell wall.

Keywords: peptidoglycan, cell wall, prophage, *Bacillus subtilis*, lytic enzymes

References
β-glucosidase and α-rhamnosidase of naringinase immobilized on sol-gel matrices: activity and stability studies in ionic liquids

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Biosynthesis is a wide application field for enzymes immobilized in sol-gel materials, including multi-enzyme biocatalysts allowing the occurrence of sequential reactions in a restricted place. Naringinase is an enzyme widely used in food and pharmaceutical industry, providing both α-L-rhamnosidase and β-D-glucosidase activities. There has been a growing interest for the health benefits of glycosides. The flavonoids, naringin and naringenin may be useful against inflammation, neurodegenerative diseases and cancer. The hydrolysis of naringin, a flavonone glycoside, with naringinase leads to reducing sugars (ramnose and glucose), to prunin and to the aglycone, naringenin [1].

Sol-gel, an innovative and economical technique was developed for naringinase immobilization in aqueous media. Higher activity and stability were obtained with naringinase encapsulated in TMOS/Glycerol. Both α-L-rhamnosidase and β-D-glucosidase, expressed by naringinase enzyme complex was evaluated in this study. Specific substrates were used: 4–nitrophenyl–α–L–rhamnopyranoside (4-NGLuc) and 4–nitrophenyl–β–D–glucopyranoside and naringinase and naringenin immobilized in sol-gel (TMOS/Glycol) was evaluated in ionic liquid systems.


Acknowledgements: Helder Vila Real is grateful to FCT for the financial support of his PhD. Grant (SFRH / BD / 30716 / 2006).

Biotechnological potential of an extracellular peroxidase from Streptomyces albus

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Chlorophenols constitute an important group of chemicals used in the synthesis of agroproducts, dyestuffs and pharmaceuticals. This widespread use of these chemicals suggest the likelihood of these compounds being present as contaminants in many environments; for example 2,4 dichlorophenol can be detected in effluents following the chlorination process during tertiary waste water treatment and in effluent following the bleaching of paper pulp. Biodegradation studies have shown that 2,4 dichlorophenol is biodegradable under aerobic and anaerobic conditions. Peroxidases (including horse-radish peroxidase) have been shown to catalyse the oxidation of 2,4 dichlorophenol and may therefore form the basis of an enzyme-based dechlorination process. The aim of this study was to assess the potential of peroxidases from a lignocellulose-degrading actinobacteria, Streptomyces albus for the degradation of chlorinated phenols.

Extracellular peroxidase activity, together with endoxylanase activity were detected during the growth of Streptomyces albus ATCC 3005 at 30°C in minimal salts media containing oat-spelt xylan as the major carbon and energy source. Maximum growth occurred after 48 h incubation with intracellular protein concentrations reaching 0.55 (± 0.06) mg ml⁻¹. Maximum extracellular xylanases activity was recorded after 96 h growth (0.33 ± 0.04 μmoles of reducing sugar ml⁻¹ min⁻¹). Similarly, maximum extracellular peroxidase activity (assessed using 2,4-dichlorophenol as substrate) was recorded after 96 h (0.34 ±0.01 U ml⁻¹), corresponding with the detection of maximum extracellular protein activity (0.43 mg ml⁻¹). Peroxidase activity was also detected when L-3,4-dihydroxyphenylalanine replaced 2,4 dichlorophenol as substrate, although activity was significantly reduced (0.06 ±0.004 U ml⁻¹ after 96 h).

Following concentration of culture supernatant by membrane filtration zymogram analysis using non-denaturing gel electrophoresis identified two major bands when either 2,4 dihlorophenol or L-dihydroxyphenylalanine was used a susbmate. To assess the biotechnological potential of the extracellular peroxidase activity, enzyme activity was assessed using a range of chlorinated substrates; 4-chlorophenol, 2,4 dichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol. Activity against all four substrates was detected, with initial activities of 0.30 (±0.05), 0.43 (±0.01), 0.45 (±0.02) and 0.18 (±0.05) U ml⁻¹ respectively. No activity was detected when heat inactivated culture supernatant was used in the assay.

The results suggest a biotechnological role for the peroxidase from Streptomyces albus in the degradation of chlorinated compounds. Further assessment of the biotechnological potential of this enzyme will require analysis of the stability of this enzyme under a range of environmental conditions.

Keywords Chlorophenols, Streptomyces albus, extracellular peroxidase.
Cardiolipin is important for *Staphylococcus aureus* to sustain the fitness in the high salinity

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*Staphylococcus aureus* is an opportunistic human pathogen that naturally inhabits our nasal cavity and skin surface. *S. aureus* is well known as salt-resistant bacterium and can grow in the presence of up to 10–15% NaCl. In 1970’s, some papers have described that its phospholipids including cardiolipin (CL) are differentially accumulated depending on the growth conditions. However, the mechanism of such phospholipids dynamics remains to be elusive. In this study, we analyzed the phospholipids change in *S. aureus* strain N315 under distinct growth phases and NaCl concentrations, using a series of mutant strains lacking genes predicted to encode CL synthases.

**Results**

i) High salt condition induced the accumulation of phosphatidylglycerophosphate (PGP). The CL content increased towards the stationary phase.

ii) The genetic analysis of two candidate genes (*cls1, cls2*) revealed that Cls2 is the dominant CL synthase, while the mutation of *cls1* alone had little effect on the CL accumulation.

iii) It was necessary to knockout both of *cls1* and *cls2* genes to abolish the CL synthesis. Our results also suggested that Cls1 has its function specifically under the high salinity.

iv) CL was necessary for full survival under high salinity or the resistance against hypertonic shock.

The possible differential utilization of the two *cls* genes will be discussed regarding their expression profiles or predicted subcellular localizations.

**Keywords** Cardiolipin, phospholipids, *Staphylococcus aureus*, high salinity

Characterization of the optimal conditions for the cultivation of "*Cladosporium cladosporioide* (Fres.) de Vries" and isolation of the pectinase produced in solid-state conditions.

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Considering the increasing use of enzymes from microorganisms in the market today, different procedures for isolation and purification are being studied. This study aimed to define the ideal time of cultivation of *Cladosporium cladosporioide* (Fr.) de Vries, for better development and production of enzymes, and partially purify the pectinases produced. To this end, we used the rice as a substrate for fungus growth. The activity of pectin methyl esterase (PME) and polygalacturonase (PG) was evaluated after a period of 10, 15 and 20 days of inoculation of the organism in the substrate, but also in each step of enzyme purification, following the method of Jen & Robinson1 and Pressey & Avants 19. The extraction of the enzyme complex was obtained by homogenization in buffer solutions at different pH (4, 5 and 6), followed by centrifugation and precipitation with ammonium sulfate saturation in 20, 40 and 60%. According to the results obtained can be observed that the micro-organism under study, the cultivation time 10 days, showed the best results of activity of polygalacturonase 105.52 (U / g min), and pectin 1480 (U / g min) in the crude extract. After 10 days of cultivation was observed stability in the growth of the colony of *Cladosporium cladosporioide* (Fr.) de Vries and reduction in enzyme activity. Similar results were found by other authors where the maximum enzyme production occurred only during cell growth, no longer detected when the culture reached the stationary phase. The decline in enzyme activity may also have been caused by a limitation in oxygen supply, since the surface of the culture was covered with a thin mycelial layer. The results also showed that the benzoate buffer pH 4.0 showed the best conditions for extraction and activity of both enzymes. This optimum pH value of reaction is included within the range found for other fungi, which typically have maximum activity in the region of acid pH. Regarding saturation with ammonium sulfate, the best results were observed in 60%, with a final yield of 108.74% for PME and 10.55% for PG and the rate of purification of 14.24 and 1.39, respectively.

**Keywords:** pectinmethyl esterase – polygalacturonase – enzyme purification
Cloning of fructansucrase gene from Weissella cibaria MBF-CNC2(1) isolated from local foods

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Fructan type of exopolysaccharide (EPS) such as inulin and its oligos (fructooligosaccharide/FOS), in addition to levan, has been well known to be used and developed in food and pharmaceutical industries. Inulin has been used widely but it is mostly produced from plant, e.g. chicory root. EPS-producing lactic acid bacteria (LAB) have been reported to produce EPS fructan, besides glucan, with characteristic as inulin as well as levan. A collection of EPS-producing LAB, isolated from various sources in Indonesia, e.g. local foods and beverages, were screened for fructansucrase (FS) gene by using degenerated PCR which primers were designed with a tag for specific cloning system. A Weissella cibaria strain MBF-CNC2(1) was shown to be potential to harbor FS gene coding for inulosucrase, an enzyme that synthesize EPS inulin. A 1690 bp DNA fragment was successfully cloned by inversed-PCR (iPCR) technique and sequenced simultaneously. By using blastx the DNA sequences analysis was obtained. Result revealed that this fragment showed DNA sequence similarity to a putative inulosucrase of L. reuteri. The cloning of this FS gene is still in the process to obtain full length gene of FS inulosucrase using iPCR.

Keywords: lactic acid bacteria, Weissella cibaria, fructansucrase, inulosucrase

Comparative study of recombinant versus natural hbFGF in survival and proliferation of primary cultured 3T3 cells

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Reproductive effects of different concentration of recombinant human basic fibroblast growth factor (hbFGF) on 3T3 Mouse embryo fibroblast cells were evaluated by their abilities to promote survival and proliferation of primary cultured cells.

In the molecule of modified hbFGF, two serines are substituted for two cysteins at positions 70 and 88 in the natural hbFGF. Both natural and modified hbFGF markedly increased the survival of primary cultured 3T3 cells. The effects were concentration-dependent, in the range of 50 ng/ml to 200 ng/ml, in similar manners for both modified and natural hbFGF. The time-course of these effects on survival was almost the same between natural and modified protein. As a result, we proved that one of the disulfide bonds that form between cysteins at positions 70 and 88 is not essential for biological activity of the basic growth factor.

Keywords: modified hbFGF, biological activity; 3T3 primary cell culture
DNA sequence-discrimination by PspGI

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PspGI is a thermostable restriction enzyme from the archaean Pyrococcus [1]. PspGI is one of a widespread family of enzymes that recognizes the sequence CCWGG in double-strand DNA and cleaves the two strands to produce fragments with 5-base, 5'-overhangs. The crystal structure of PspGI bound to its substrate DNA has been solved [2]. The enzyme acts as a homodimer, encircling the DNA and contacting the base pairs in both the major- and the minor-grooves. We are investigating the molecular mechanism by which PspGI recognizes the central base pair in its sequence. From the crystal structure, recognition of the C-G base pairs in each half of the sequence occurs by major-groove contacts with the conserved amino acids Arg164, Glu165, and Arg166 (R-E-R) in each subunit, but the mechanism for recognizing the central base pair—which can be either A:T or T:A (i.e. W:W) but not G:C or C:G (i.e. not S:S)—is unclear and remains the subject of experimentation [3]. We tested whether this discrimination occurs passively, by steric clash in the minor groove. A:T and T:A base pairs are smaller than G:C and C:G base pairs in the minor groove due to the presence of the 2-amino group that protrudes from the ring of Guanine but not from Adenine. If the space in the DNA-binding site of PspGI needed to accommodate this 2-amino group were occupied by an amino acid instead, then G:C and C:G would not fit into the site, whereas A:T and T:A would fit. From the crystal structure, two amino acids that could potentially clash with Guanine in this way were identified: Tyr67 and Phe97. Each was changed to other amino acids by mutagenesis, and the resulting variant enzymes were assayed for cleavage of CCSGG in addition to CCWGG. The work is on-going. All of the catalytically active Phε97 mutants examined to date continue to cleave only CCWGG, suggesting that Phe97 is not in fact involved in sequence-discrimination. The Tyr67 mutants also continue to cleave only CCWGG, but many now appear to bind—although not to cleave—CCSGG as well, suggesting that Tyr67 might indeed be a factor in sequence-discrimination,


Keywords Restriction enzyme; PspGI; DNA sequence-specificity; minor groove; base-flipping

Effect of different carbon sources used for Candida guilliermondii precultivation on Xylose reductase and Xylitol dehydrogenase activities during sugarcane bagasse hemicellulosic hydrolysate fermentation

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Xylitol is a sugar-alcohol used in many clinical applications and it can be obtained from yeast xylose metabolism. The xylose is the main pentose sugar in the hemicellulosic hydrolysates, which contains besides sugars, toxic compounds released during the diluted acid hydrolysis of lignocellulosic materials. The enzymes xylose reductase (XR) (EC 1.1.1.21) and xylitol dehydrogenase (XDH) (EC 1.1.1.9) take part in xylose-to-xylitol biocconversion. In the case of the utilization of hemicellulosic hydrolysates the phenolic are the main inhibitory compounds of the enzymatic activities and detoxification procedures are necessary to reduce the concentration of these compounds. This work evaluated the effect of different carbon sources used for Candida guilliermondii precultivation on xylene reductase and xylitol dehydrogenase activities during sugarcane bagasse hemicellulosic hydrolysate fermentation. Initially the hydrolysate (pH ~1.52) was vacuum evaporated to increase its sugar concentration to 80 g/L. Since phenolic compounds were also increased during this procedure it was necessary the detoxification of the hydrolysate by ion exchange resin (A-860S; A500PS and C-150-Purelite 6). The resin treatment (resin:hydrolysate ratio 1:2 v/v) was carried out in Erlenmeyer flasks (2000 mL) at 200 rpm, 30 °C for 1h. The xylitol production was evaluated by using cells previously grown in different carbon sources (g/L): xylose (30.0), glucose (30.0) and/or a mixture of both sugars (xylose-30.0 and glucose-2.0). All experiments were carried out in Erlenmeyer flasks (125 mL) containing 50 mL of medium at 200 rpm, 30 °C. The incubation times for inoculum and fermentation experiments were 24 and 72 h, respectively. In all experiments, the media (g/L): xylose-75.0; glucose-4.9; arabinose-4.4; acetic acid-1.9 and phenolic compounds -1.0) were supplemented with the following nutrients (g/L): ammonium sulfate (2.0), calcium chloride (0.1) and rice bran extract (20.0). The fermentation was performed in triplicate using 1.0 g/L initial cell concentration and 5.5 pH. The sugars and acetic acid concentrations were determined by liquid chromatography, while total phenolic compounds and enzymatic assays were determined by spectrophotometry. The resin treatment removed about 93% of total phenolic compounds from sugarcane bagasse hemicellulosic hydrolysate. In the experiments with when cells precultured on the glucose and xylose mixture the XR (EC 1.1.1.21) was favored. In the experiments with cells precultivated on the xylose and glucose mixture the XR activity (0.499 U/mg Prot) was favored, while the XDH activity (0.703 U/mgProt) and cell growth (10.6 g/L) were improved when the medium containing only glucose as carbon source. However, in relation to the xylitol yield and volumetric productivity, it was observed that these fermentative parameters were favored when only xylose as carbon source was employed in the medium, which corresponded to the maximum xylitol consumption (81%) after 72h of fermentation. Under this condition it was observed that cells were able to consume 100% of the acetic acid present in the medium.

Keywords sugarcane bagasse hemicellulosic hydrolysate; xylose reductase; xylitol dehydrogenase and Candida guilliermondii

Acknowledgments: FAPESP, CNPq and CAPES.
Effect of soy lecithin on the ligninolytic enzymes production by the white-rot fungus *Anthracophyllum discolor*

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Natural and synthetic surfactant has the potential to enhance the bioavailability of hydrophobic organic pollutants in contaminated sites. Interestingly, it has also been proposed that these surfactants can be used as a growth substrate, resulting in an increased microbial biomass, thereby promoting a more efficient bioremediation process. Biosurfactants are less toxic and more environmentally benign than synthetic surfactant. However, some of these compounds can be toxic, recalcitrant and exert inhibitory effects on the biodegrading microorganism. Until now, most studies have been conducted mainly to evaluate the effects of chemical surfactant on bacteria, and some white-rot fungi, as *Phanerochaete chrysosporium* and *Trametes versicolor*. But few researchers have addressed to analyze the effects of phytogenic surfactant on the ligninolytic activity of white-rot fungus. In this context, the aim of this study was to evaluate the effect of soy lecithin (SL) on the production of ligninolytic enzymes, laccase (Lac), lignin peroxidase (LiP), manganese peroxidase (MnP), and manganese independent peroxidase (MiP), produced by the white-rot fungus *Anthracophyllum discolor*.

Previous Studies have demonstrated that the optimum culture conditions of *A. discolor* for maximizing ligninolytic enzyme production in modified Kirk medium were 26ºC; pH 5.5; and C/N ratio of 250, therefore, this study was done under these optimized conditions. The modified Kirk medium was supplemented with soy lecithin in the range 0 to 1 gL⁻¹. The flasks with 50 ml of medium inoculated with one agar plugs of *A. discolor* previously cultivated on glucose malt extract agar, were incubated for 31 days in stationary cultivation, and the ligninolytic activity in the extracellular fluid was evaluated periodically.

The results showed that *A. discolor* predominantly produced MnP in the evaluated concentrations of soy lecithin, with a maximum activity of 30.64 UL⁻¹ ± 4.61 UL⁻¹ when the medium was supplemented with 1 gL⁻¹ of soy lecithin after 23 days of incubation. On the other hand, LiP presents a maximum of 30.11 UL⁻¹ ± 0.13 UL⁻¹ with 30 days of culture, when the medium was supplemented with 0.15 and 0.30 gL⁻¹ of soy lecithin. While the amount of MnP produced did not exceed 5 UL⁻¹.

**Keywords:** *A. discolor*, soy lecithin, ligninolytic enzymes

**Acknowledgements:** Investigation financed partially by Fondecyt 1090678

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Engineering novel DNA binding specificity in Type II restriction endonucleases.

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Since the binding of proteins to discrete DNA sequences is fundamental to many biological processes, the ability to design and generate proteins that specifically bind at DNA sequences of choice is highly desirable. Here we present a methodology that has enabled us to engineer DNA binding proteins that bind and act at new, rationally chosen recognition sequences (1). This specificity engineering has been performed with a subgroup of the Type II restriction enzymes, proteins that exhibit exquisite sequence specificity. We recently identified and characterized a family of Type II restriction endonucleases that bind and cut at different sequences, yet share highly similar protein sequences. This sequence similarity allows accurate alignment of the protein sequences, while the presence of a common adenine that is methylated by the enzymes allows the recognition sequences to be accurately aligned. Correlations between the aligned amino acid residues and the base pair present at any given position in the aligned recognition sequences are then observed. This bioinformatic approach allowed the identification of the amino acid pairs that specify DNA base recognition at three positions in the recognition sequence. By altering the amino acids at these identified positions to those correlated with recognition of a desired new base, enzymes that recognize and cut at predictable new DNA sequences were created. The enzymes so altered have similar specific activity compared to the wild type enzymes. Using simple and predictable mutagenesis we demonstrate that it is possible to create hundreds of unique new Type II restriction endonuclease specificities. We anticipate that the methodology described should also be applicable to other families of DNA binding proteins.

**Keywords** DNA binding protein, DNA specificity engineering, restriction endonuclease

Enzymatic characterization of N-terminally truncated dextran transglucosidase cloned from *Leuconostoc citreum* HJ-P4

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Dextran transglucosidase (DxSGase; EC 2.4.1.5) is one of glucosyltransferases, belonging to the glycoside-hydrolase (GH) family 70 that catalyzes synthesis of a high molecular weight polymer, dextran, from sucrose. Commonly DxSGases have been found from lactic acid bacteria, mainly strains of *Leuconostoc mesenteroides*. In general, DxSGases have very huge molecular mass over 160 kDa, which causes extremely low expression level in *E. coli* system.

In previous report, a dextran transglucosidase (DxSGase) gene from *Leu. citreum* HJ-P4 has been amplified and cloned in *E. coli*. The DxSGase gene consists of 4,431 nucleotides encoding 1,477 amino acid residues sharing 63~98% of amino acid sequence identities with other known DxSGases from *Leu. mesenteroides*. Interestingly, 0.1 mM of IPTG induction at 15°C remarkably increased the DxSGase productivity, which was over 330-fold higher than that induced at 37°C. Same as the native enzyme, recombinant DxSGase could successfully produce a series of isoamyloligosaccharides from sucrose and maltose, on the basis of its transglycosylation activity.

Even though DxSGases are very important in dextran synthesis, however, extremely low productivity of DxSGase can be the great barrier in their industrial applications. Therefore, more compact and minimized DxSGases with much lower molecular mass than wild-type enzyme should be developed for the increased productivity and stability.

In order to reduce the molecular mass of DxSGase, its gene was continuously truncated by step-wise degradation using exonuclease III. For the simple detection of the truncated DxSGase, high-throughput screening techniques should be developed first. To screen the DxSGase mutant with dextran-forming activity, *E. coli* transformants were tooth-picked onto LB agar medium containing sucrose. After incubation for 6~8 hours, soft the agar solution with D-cycloserine was overlaid on the plate. During incubation at 30°C for 12 hours, positive clones were successfully selected from their capability dextran-forming around the colony on a plate. The high-throughput screening method via direct detection of polymer formation on agar plate developed here has been successfully applied to the screening of various active truncated mutants.

N-terminal truncation of DxSGase has been tried to generate the minimized mutant enzymes with considerable activity. Each positive clone was confirmed by restriction enzyme treatment and DNA sequencing analysis. Primary structure of truncated mutants was analyzed and compared with each other. On the basis of DxSGase activity, considerably active 6 clones, LcDS Δ42, Δ80, Δ92, Δ95, Δ194, and Δ199, were finally selected and each mutant was characterized. The most compact and minimized mutant, LcDS Δ199, truncated its nucleotides of 597 bp (199 amino acids), still has considerable specific activity and transglycosylation activity to produce isoamyloligosaccharides (IMOs) from sucrose and maltose acceptor. LcDS can produce IMOs in various length, including mainly panose, on the basis of its transglycosylation activity. Moreover, its expression level and recovery yield was improved compared to LcDS wild-type. The resulting truncated mutants, therefore, can be used as more efficient biocatalysts for the industrial synthesis of dextran polymers or transglycosylation of bioactive natural compounds.

**Keywords** dextranase; N-terminal dextran; truncation; enzymatic characterization; transglycosylation

Enzymatic properties and expression levels of various chimeric enzymes between barley alpha-amylase isozyme 1 and 2 in *E. coli*

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Alpha-amylases (EC 3.2.1.1) are retaining enzymes of Glycoside Hydrolase Family 13 (GH-13) acting on internal alpha-(1,4)-glycosidic linkages in starch materials and occur widely in bacteria, fungi, animals, and plants. Barley (*Hordeum vulgare*) malt contains two alpha-amylase isozymes, AMY1 and AMY2 readily distinguished by their isoelectric points of AMY1 (low pI = 4.7~4.9) and AMY2 (high pI = 5.9~6.1). Although barley AMYs share up to 80% amino acid sequence identity and almost identical three-dimensional structure, their enzymatic properties differ remarkably. AMY1 has higher affinity for calcium ions than AMY2 and only AMY2 is strongly inhibited by the endogenous barley alpha-amylase/subtilisin inhibitor (BASI). In addition AMY2 dominates in barley malt, but is poorly expressed in *Saccharomyces cerevisiae* and *Pichia pastoris* compared to AMY1.

Despite the well-known three-dimensional structure of both AMY isozymes and a large amount of available biochemical data, the features that cause major differences in isozyme properties remain unclear. Therefore, the comparative investigation of these isozymes may be one of the good candidates to elucidate the relationships between structure and function of proteins. To date, however, both AMY genes have been poorly expressed in recombinant *E. coli* systems, which has been a major barrier in enzyme engineering approaches for barley AMYs.

Interestingly, it was revealed that their expression can significantly be affected by induction temperature. The induction of AMYs at 15°C remarkably increased their expression level in *E. coli*, while extremely low productivity at 37°C. As a result, high amount of recombinant AMYs with C-terminal six-histidines were successfully expressed under the control of inducible T7 RNA polymerase promoter in pET-21a. Even though their expression levels highly increased in *E. coli*, the productivity of AMY2 still remains much lower than that of AMY1.

According to the previous report, the 42nd alanine residue located within the (β/α)8 loop of the catalytic (β/α)8-barrel domain may be important in the secretion of AMY in *S. cerevisiae*. In the present study, expression levels of AMY2 mutant A42P and wild-type AMY2 at 15°C were compared under the control of IPTG-inducible T7 RNA polymerase promoter. As a result, about 10-times higher amount of recombinant AMY2 A42P was obtained and simply purified via Ni-NTA chromatography.

In order to improve the expression of AMY2, chimeric AMYs between AMY1 and 2 were constructed and their expression levels and enzymatic properties were characterized. Based on the sequence alignment between AMY1 and AMY2, the structural gene regions were finally divided into eight modules encoding the following stretches; (1) A..A 1-90, (2) A.A. 91-160, (3) A.A. 161-205, (4) A.A. 206-239, (5) A.A. 240-264, (6) A..A. 265-295, (7) A.A. 296-318, (8) A..A. 319-414 in AMY1/ 403 in AMY2. Recombinant AMYs were purified via Ni-NTA chromatography and its calcium-dependent hydrolyzing activities including hydrolysis patterns on various starch-derivatives were comparatively examined with each other. The shuffled chimeric enzymes were categorized into three groups (i) AMY1-type (e.g. AMY1112 and AMY-C6), ii) AMY2-type (e.g. AMY2221, AMY11221, AMY12221, and AMY-E10), and iii) the mixed/intermediate-type (the remaining chimeras). The results indicate that subtle amino acid differences in positions flanking calcium-binding residues elicit striking differences in the calcium dependence of the enzymatic activity.

**Keywords** barley α-amylase isozymes; chimeric enzymes; expression level; enzymatic characterization
Enzymatic properties of various hemicellulose-hydrolyzing enzymes cloned from a hyperthermophile of Thermotoga neapolitana

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Hemicelluloses are the matrix polysaccharides of the plant cell wall and the most abundant source of renewable polymers on the earth. A number of versatile and specific enzymes are involved in the complete degradation of these polymeric substrates. Especially, the efficient utilization of xylans as sources of bio-fuels and industrial chemicals requires total understanding of the enzyme systems for their conversion. Due to the high complexity and structural variability of heteroxylans, their enzymatic hydrolysis can be achieved via the concerted treatment of various hydrodrolases that include the main chain-cleaving enzymes, endo-β-(1,4)-xylanases (EC 3.2.1.8) and β-xylanases (EC 3.2.1.37), and the side chain-active enzymes, acetyl-xylan esterases (EC 3.1.1.72), α-glucuronidases (EC 3.2.1.139), ferulic acid esterases (EC 3.1.1.73), and α-L-arabinofuranosidases (EC 3.2.1.55).

For high conversion yield of cellulose biomasses to ethanol, more efficient enzymatic saccharification should be followed by alcohol fermentation. The hydrolysis reactions at high temperature generally increase the saccharification yield, compared to those by mesophilic hydrodrolases. Development of highly thermostable hemicellulose-degrading enzyme systems will be one of the best ways to improve the bioethanol productivity.

Thermotoga neapolitana, a hyperthermophile, is known as a vegetarian bacterium, because it possesses a number of carbohydrate-active enzymes in its genome. Except ferulic acid esterase, a variety of hemicellulose-hydrolyases genes and some cellulosytic enzyme genes including β-glucosidases have been found. In this study, five core enzyme genes including endo-β-(1,4)-xylanase (TnXN), β-xylanase (TnXN), acetyl-xylan esterases (TnXAE), α-glucuronidases (TnGU), and α-L-arabinofuranosidase (TnAFA), were successfully cloned from T. neapolitana DSM 4359. PCR-amplified DNA fragment containing each gene was cleaved with combination of appropriate restriction enzymes and ligated into a constitutive expression vector of pHCXHD with a C-terminal six-histidines fusion tag. Each enzyme gene has an open-reading fram coding 347 (TnXN), 558 (TnGU), and 512 (TnAFA) amino acids, respectively. The predicted molecular masses range from 37,000 to 87,000 Da. The recombinant enzymes were expressed in E. coli MC1061 and efficiently purified to a homogeneity via heat treatment at 60°C for 1 hour followed by a boiling step with 2% H2O2. Results obtained showed a 10% decrease in Kappa number, a 3.5 % increase in ISO brightness and a remarkable saving in H2O2 consumption. On the other hand, application of LMS to decolourise textile dyes requires non-chromogenic mediators and up to date best results were obtained with phenolic compounds related with lignin. For this study, we found that 0.1 mM acetosyringone was the best mediator to decolourise the azo-type dye Reactive Green acting together with 300 mU laccase. With this LMS, a 90% decolourization was achieved. Moreover, this enzyme retains the most of its activity in the presence of high concentrations of different salts and specific inhibitors such as sodium azide. Finally, analysis of toxicity after the treatment (Microtox® System) also showed a high degree of detoxification (increase in EC of 100% compared with control).

Keywords Thermotoga neapolitana, α-L-arabinofuranosidase; acetyl xylan esterase; α-glucuronidase; β-xylanase; β-glucosidase

Exploring the biotechnological applications of an halotolerant pH-versatile laccase produced by Streptomyces ipomoeae CECT 3341

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Laccases (EC 1.10.3.2) are multicopper oxidases with a large catalytic versatility due to their low substrate specificity against phenolic compounds and aromatic amines. During last years, these enzymes have received great attention for biotechnological applications. In fact, the enhancement of its oxidation capability throughout the action of redox mediators [1] allows the development of new strategies for degradation of xenobiotics compounds, pulp delignification, textile dyes bleaching, etc [2-6]. Although laccase and laccase-mediated systems have been studied in white-rot fungi for a wide range of applications, nowadays bacterial laccases are being considered to be applied for biotechnological purposes because their physico-chemical and kinetics characteristics. The most remarkable feature of these laccases is the wide range of pH in which they are active in comparison with the acidic pH of reaction characteristic of most of the fungal laccases [5-8]. This pH versatility could fulfill the industrial requirements for full application of these enzymes in biotechnological processes. In our group, the oxidative enzymatic mechanisms involved in lignocellulose degradation by other ligninolytic microorganisms such as Streptomyces have been studied. The oxidative capability of these actinobacteria lays mainly in the production of laccases. One of them produced by Streptomyces cyanus CECT 3335 has shown its suitability for the biobleaching of eucalyptus kraft pulp [5]. More recently, a new laccase produced by S. ipomoea CECT 3341 has been purified and characterized showing some different physico-chemical characteristics from that produced by S. cyanus. In fact, substrate specificity of this laccase depends on the pH, (i.e. optimal pH for ABTS or phenolic compounds are 4.5 or 8, respectively). In the present work we screen the potential application of the laccase produced by S. ipomoea and different mediators (Laccase-Mediator System or LMS) for the biobleaching of eucalyptus kraft pulp and for decolourisation and detoxification of a textile azo-type dye.

The treatment of eucalyptus kraft pulp was carried out with 300 mU laccase per gram of pulp in the presence of 1 mM ABTS or phenolic compounds and for decolourisation and detoxification of a textile azo-type dye. The treatment of eucalyptus kraft pulp was carried out with 300 mU laccase per gram of pulp in the presence of 1 mM ABTS as mediator in acetic buffer pH 4.5 to get a 10% (w/v) consistency. Enzyme treatment was maintained at 60°C for 1 hour followed by a bleaching step with 2% H2O2. Results obtained showed a 10% decrease in Kappa number, a 3.5 % increase in ISO brightness and a remarkable saving in H2O2 consumption. On the other hand, application of LMS to decolourise textile dyes requires non-chromogenic mediators and up to date best results were obtained with phenolic compounds related with lignin. For this study, we found that 0.1 mM acetosyringone was the best mediator to decolourise the azo-type dye Reactive Green acting together with 300 mU laccase. With this LMS, a 90% decolourization was achieved. Moreover, this enzyme retains the most of its activity in the presence of high concentrations of different salts and specific inhibitors such as sodium azide. Finally, analysis of toxicity after the treatment (Microtox® System) also showed a high degree of detoxification (increase in EC of 100% compared with control).

Keywords Laccase, Streptomyces, Biobleaching kraft pulp, Detoxification, Decolorisation azo dye

References

Keywords Laccase, Streptomyces, Biobleaching kraft pulp, Detoxification, Decolorisation azo dye
Expression of keratinase gene in *Bacillus megaterium* using an expression vector of pHIS1525.SPAPA and utilization of the resulting recombinant strain for chicken feather degradation prior to biogas production

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An increasing quantity of chickens is being utilized annually in the poultry industry, producing a huge volume of chicken feather waste which presents a high quality supply of keratin. Keratinases possessing high level of keratinolytic activity on insoluble keratin play a crucial role in hydrolyzing chicken feathers. Ever since the discovery of proteolytic ability as well as water solubility of keratinase, many industrial processes regarding keratinase application have been developed. A recently invented application to handle poultry waste is to utilize feathers for biogas production. Obviously, large amount of keratinase is required to break down the keratin prior to further conversion to biogas. Previously, several researches have shown that certain bacteria are able to produce keratinase but it is still a challenge to find out which bacteria is the most reliable source for the production with high efficiency. These challenges gave rise to the molecular biologists to bring the focus on gene cloning to develop recombinant strains resulting in overproduction of keratinase. Over the course of various cloning and expression experiments of similar proteins, it was found that *Bacillus megaterium* could be a susceptible host cell for keratinase production.

In our study, the keratinase gene from the chromosomal DNA of *Bacillus licheniformis* ATCC®53757 was PCR amplified and subsequently cloned into *Bacillus megaterium* expression vector, pHIS1525.SPAPA. *Bacillus megaterium* ATCC®14945 strain was transformed with the recombinant plasmid, pKHERHS1525.SPAPA. The KER gene was expressed under xylose inducible promoter, and the product was then purified using Ni-NTA affinity chromatography. After 18 h of incubation an extracellular keratinase activity of 29U ml⁻¹ was achieved (one unit of activity was determined as the amount of enzyme required to an increase of 0.01 in A420 after 30 min of incubation at 37°C). The recombinant strain was further examined for feather degradation using intact chicken feather waste as carbon source. The chopped chicken feathers were partially degraded by the recombinant strain after three days of incubation and the total macroscopic digestion was ultimately observed after seven days resulting in a yellowish peptide rich fermentation broth. The biogas potential of the hydrolysate will be compared with that of untreated feathers by performing anaerobic batch digestion experiments.

Keywords: *Bacillus licheniformis, Bacillus megaterium*, Gene expression, Keratinase, Feather degradation, Biogas production

Fed-batch fermentation for heterologous protein production by recombinant *Pichia pastoris*

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*Pichia pastoris* is a methylo trophic yeast widely used for heterologous recombinant protein production. This yeast has potential for high level expression, efficient secretion and growth to very high cell densities. Fed-batch fermentation has been widely used to enhance protein production by *P. pastoris*. Frutalin is a α-D-galactose-binding lectin isolated from *Arctocarpus incisa* seeds, successfully used as a cancer diagnostic tool and thus its large-scale production is aimed. This lectin has been previously expressed and produced in *P. pastoris* using a batch process. Therefore, the present work aims at evaluating a fed-batch fermentation process as an alternative to improve the production of recombinant frutalin by *P. pastoris* KM71H.

Cultivations were carried out in a 1.6 L reactor, in three distinct phases: 1) initial batch fermentation for cells growth in BMGH medium; 2) a fed-batch phase with 50% glycerol and 12 mL/L of trace metal solution; 3) a fed-batch phase where cells were induced by 0.5% methanol and 12 mL/L of trace metal solution. During the fermentation, the dissolved oxygen was kept above 30% saturation, aeration ratio was fixed at 1.5 vvm, and pH values were controlled at 5.0. In the first and second fermentation phases, the temperature was maintained at 30 °C, being decreased to 21 °C at the end of second stage. Total cell concentration was determined by measuring the absorbance of the broth at 600 nm, while glycerol consumption and methanol concentrations were detected by HPLC. The recombinant frutalin production was detected by denaturing SDS-PAGE, being the bands visualized by staining with Coomassie Brilliant Blue R250. The lectin activity was checked by hemagglutination assays towards rabbit red blood cells.

High cell density (98.8 g/L dry weight) was obtained during the fed-batch process (Fig. 1A), which is generally desirable since the concentration of secreted protein in the medium often increases proportionally to the cell density. Analysis by SDS-PAGE showed frutalin production at 120, 132 and 144 h. Native frutalin migrates in SDS-PAGE as a double band, where the upper band corresponds to the glycosylated isoforms and the lower band to the non-glycosylated isoforms. Recombinant frutalin migrated in gel as a single band (Fig. 1B) and exhibited hemagglutinating activity towards rabbit erythrocytes. Optimization of the induction phase is still on course. Nevertheless the results obtained so far show the feasibility of the fed-batch process for large-scale recombinant frutalin production by *P. pastoris* KM71H. Supported by: CNPq, FAPESP, ERASMUS.

Fig. 1. Production of recombinant frutalin in fed-batch fermentation process by *P. pastoris* KM71H. A) Yeast growth profile and time course of batch, fed-batch and induction phases. B) SDS-PAGE analysis of the supernatant at induction phase.
Gene cloning, Expression and Characterization of Thermo-Acid Stable Recombinant Phytase from Mycobacterium smegmatis

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Phytase, also known as phytate-degrading enzyme, catalyzes the hydrolysis of phytate (inositol hexakisphosphate) with sequential release of phosphate and lower inositol phosphate. Non-ruminant animals such chicken cannot effectively use the 6 phosphates esterified to inositol and phytate-bound nutrients present in their diet because there is little or no phytase activity in their digestive tract. Inorganic phosphates, if supplemented in the animal feed formulation, result to excess excretion of phosphorus to the environment which will eventually lead to environmental pollution. The addition of phytase into animal feed formulation is the best strategy to cater this problem. Recently, microbes become the potential and more feasible sources of phytase. In the present work the gene encoding for the novel phytase from Mycobacterium smegmatis has been amplified from the genomic DNA by the polymerase chain reaction (PCR) methodology. The purified amplified gene cloned into pBAD-TOPO and transform into Escherichia coli for protein expression. The recombinant phytase was purified to homogenity and biochemically characterized with respect to its molecular characterization, specific activity, pH activity profile and substrate specificity. The characterization shows the recombinant phytase from M. smegmatis is highly thermostable and resistant to the extreme pH conditions. These properties give it a potential application in animal feed industry.

Keywords Phytase; Phytate; Mycobacterium smegmatis; Thermostable; pH resistant

Hemicellulolytic and cellulolytic activities in the culture filtrate of Thermoascus aurantiacus ATCC 204492 induced by products of xylan hydrolysis

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Thermoascus aurantiacus can secrete most of the hemicellulolytic and cellulolytic enzymes, however, endo-xylanase is the main enzyme detected in its culture. Significant levels of xylanase were detected with various lignocellulosic materials. In order to establish the inducers of xylanase in T. aurantiacus the mycelia was grown in glucose up to the late exponential phase of growth, washed and suspended in fresh medium not supplied with a carbon source. Pre-weighted amounts of xylene (final concentration of 3.5 mg/ml), xylose (7 mg/ml) and hydrolyzed xylan from sugarcane bagasse containing xylose, xylobiose and xylotriose (HXSb) (6.8 mg/ml) were tested as inducers of xylanase. The mixtures were incubated on a rotary shaker at 45 °C and at various times, aliquots were taken and centrifuged and activity of hemicellulases and cellulases were determined in the supernatant. Xylanase was induced by all the three carbon sources tested. Xylobiose showed the faster induction with the enzyme production stopping after ten hours, even at low carbon source consumption; therefore xylobiose appears to be the natural inducer of xylanase. The xylanase induction in xylose continued until 25 h, even after xylose exhaustion. Only negligible xylanase activity was determined in the mixtures supplied with HXSb. Xylose present in HXSb was consumed in the first ten hours and xylobiose or xylotriose was hydrolyzed very slowly. B-xylosidase was not induced by xylose, and hence no transxylosidase activity must exist, which enables to form products of higher molecular weight than xylose. Arabinoarabinofuranosidase were also induced by all the inducers tested. The profile of arabinoarabinofuranosidase induction was very similar in xylose or HXSb, but xylose showed some positive effects also. The production of xylanases seems to be accompanied by production of cellulolytic enzymes independently on the carbon source used as the grow support; however we can also speculate that xylanase acts as endoglucanase. These results evidence that these enzymes possess certain substrate cross-specificity.

Keywords: xylanase, cellulase, Thermoascus aurantiacus, induction, xylobiose.
Heterologous expression of biotechnologically important enzymes from basidiomycetes

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White-rot fungi as a potent source of unique hydrolases and oxidoreductases are currently of special interest for different areas such as feed, food, pharmaceutical, and cosmetics industries. The lipase Lip2 from *Pleurotus sapidus* is one of few enzymes catalysing the hydrolysis of xanthophyll esters to free xanthophylls (Fig. 1A) which are widely used as colorants and antioxidants. Free xanthophylls are currently liberated from their ester precursors using hot (80°C) and concentrated sodium hydroxide solution which results in the formation of side products and imposes high requirements concerning safety issues and waste disposal. This has stimulated the search for biotechnological options.

The new extracellular peroxidase MsP2 from *Marasmius scorodonius* catalyses the degradation of carotenoids (Fig. 1B) and belongs to the rare group of the dye-decolorizing peroxidases produced by higher fungi. The low expression levels of such lignolytic enzymes limit their isolation, characterisation, and technical application.

The genes encoding Lip2 from *P. sapidus* and MsP2 from *M. scorodonius* were expressed in different *Escherichia coli* strains using pET (cytoplasmic expression), pBAD (periplasmic expression) and pCold (cold shock induced expression) vectors. A fusion with a C-terminal His-tag was used for purification and immunochemical detection of the target proteins. The expression of Lip2 and MsP2 led to the production of recombinant proteins, mainly as inclusion bodies. Refolding strategies and periplasmic (Lip2) or cold shock induced (MsP2) expression have delivered the catalytic active enzymes.

Keywords: basidiomycete; *Escherichia coli*; heterologous expression; in vitro refolding; lipase; peroxidase.

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Hydrolysis of pretreated bagasse with the thermophilic enzyme mixture of *Thermoascus aurantiacus*

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The thermophilic fungus *Thermoascus aurantiacus* has shown the capacity to grow on a wide variety of substrates producing cellulolytic and hemicellulolytic enzymes which present potential advantages in lignocellulose hydrolysis. Thermostable enzymes are produced with high specific activity and stability. *T. aurantiacus* was cultivated on four different types of milled agricultural residues: sugarcane bagasse, sugarcane straw, wheat straw and corn cob. After determined periods of time xylanase, endoglucanase, exoglucanase, β-glucosidase and β-xylosidase activities were evaluated. Xylanase was produced in all substrates, with much more expressive activity than cellulases, indicating that this fungus presents more xylanolytic than cellulolytic profile. The highest level of xylanase was determined in the medium containing sugarcane straw at 9 days (1679.8 U/g) and the β-glucosidase (29.9 U/g) at 6 days. The highest endoglucanase was produced on sugarcane bagasse (108.9 U/g) at 9 days. With inoculum load of 10⁸ ascospores/g the amount of exoglucanase activity exceeds 10 times that produced with 10⁴ ascospores/g of dry sugarcane bagasse. The activities of the other enzymes did not change. The purification and characterization of candidate enzymes for sugarcane bagasse hydrolysis were performed in an ion exchange column, DEAE Sepharose CL6B, and a xylanase of 31.5 kDa, an endoglucanase of 32.4 kDa and a β-glucosidase of 76.3 kDa were isolated. Evaluation of the most interesting protein using characterized mixtures of purified proteins on pretreated alkaline sugar cane bagasse showed that the raw material and pretreatment affected the enzymatic hydrolysis.

Keywords: xylanase, cellulase, *Thermoascus aurantiacus*, purification, enzymatic hydrolysis.
Lignin degradation by complex pellets of white-rot fungus *Anthracophyllum discolor* in airlift reactor

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The white-rot fungi (WRF) have been studied because are organisms able to tolerate and degrade higher concentrations of diverse persistent organic compounds. The ability of WRF to degrade pollutants has been attributed to the action of non-specific extracellular ligninolytic enzyme system, composed principally of laccases, lignin peroxidases (LiP) and manganese peroxidases (MnP).

The WRF can be grown in submerged cultures by several different morphological forms: suspended mycelia, clumps or pellets. However, pellet form becomes the desirable morphology for industrial processes, because this form not only increases the yield but also allows a better biological control of fungus growth and other operational characteristics. The benefits of using pellets are: higher enzyme production, lower oxygen limitation and better hygienic conditions, among others. Production of laccase has been widely reported in fungi, plants, and insects and quite often these enzymes are produced as multiple isozymes. Currently, efforts are being made worldwide to find new strains capable of producing of high levels of lignin degrading enzymes because of their potential application in: biotransformation of lignocellulosic biomass into fuels and chemicals, biopulping and biobleaching of paper pulp, and azo and thio dyes decolorization.

In this context, pellets of white-rot fungus *Anthracophyllum discolor* with carbon activated and sawdust (complex pellets) were obtained in airlift reactor. The complex pellets were formed by a center of activated carbon and sawdust surrounded by fungal mycelium, at difference of simple pellets (only mycelium), that are hollow sphere (Figure 1).

Keywords: Laccase, *Trametes* sp., soy meal medium, induction.

Laccase Production by Basidiomycetes Isolated from Forest in Nuevo León, México

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The white-rot fungi have shown to produce a powerful battery of extracellular enzymes that can degrade lignin. Production of Laccase has been widely reported in fungi, plants, and insects and quite often these enzymes are produced as multiple isozymes. Currently, efforts are being made worldwide to find new strains capable of producing of high levels of lignin degrading enzymes because of their potential application in: biotransformation of lignocellulosic biomass into fuels and chemicals, biopulping and biobleaching of paper pulp, and azo and thalocyanine dye decolorization. Many studies of ligninolytic enzymes production in white-rot fungi have been carried out in liquid medium, but the enzyme production levels varied to much even between strains of the same species. Due this, in the present work, a soy meal medium of high nitrogen content was tested for ligninolytic enzymes production by four strains isolated from forest of Northern Nuevo Leon State in Mexico, named *Trametes villosa* (RVAN18), *Schizophyllum commune* (RVAN19), and two *Trametes* spp strains (RAVAN2 and RYAN12), and *Pleurotus ostreatus* ATCC58053 (Po). A preliminary assay was done in solid media with different chromophores, ABTS, o-adisidine and guaiacol. A total of five strains, four natives and one control strain, showed an extracellular oxidases and peroxidases production in Petri dishes. RVAN2 and RYAN12 were the fungus with maximum oxidase and peroxidase activity following by Po and RVAN19. In addition, constitutive levels of Lac, LiP and MnP were analyzed in a glucose containing mineral medium (GMM), having low nitrogen content and in SMM to examined the effect of high nitrogen in the production of the same enzymes. In GMM, the level of laccase was 0.69 U/mL in RVAN12, followed by Po with 0.061 U/mL and RVAN2 with 0.048 U/mL. By the other way, SMM enhanced the ligninolytic activity in four strains, but only the strains RVAN2 and RYAN12, were capable to produce the three enzymes in this medium. The highest levels of laccase were 5.1 U/mL (RVAN2) and 13.4 U/mL (RVAN12), superior or similar than the collection strain *Pleurotus ostreatus* ATCC 58053. The crude extract of the RVAN2 and RVAN12 showed two isoenzymes in the complex medium indicating that SMM induce the synthesis of isozyme in RVAN2 and RVAN12, because in GMM only constitutive laccase is present. Finally, even though the MnP levels were low with respect to others activities, the fact that RVAN2 and RVAN12 produce high levels of Lac and LiP, respectively, more than *P. ostreatus* ATCC 58053, suggest that these strains could be candidates for further studies focused in bioremediation studies, mainly dye decolorization, biobleaching, detoxification, and other processes. However, more specific studies are necessary to characterize the isozyme produced in SMM and increased their activity by the use of inducer allow to use them in different bioremediation and industrial processes, and mainly the necessity of continuous searching for novel strains.

Keywords: Laccase, *Trametes* sp., soy meal medium, induction.
Microbial lipase purification using aqueous two-phase system

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Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) catalyze different reaction such as hydrolysis, inter-esterification, esterification, alcoholysis, acidolysis, and aminolysis. Many microorganisms are able to produce lipolytic enzymes commercially significant. However, the industrial use requires the application of purification methods for the separation of enzyme. Aqueous two-phase system is a great alternative to purification because it is less aggressive for enzymatic process. This work discuss the application of aqueous two-phase system in purification of lipases (6351.91 U/mL) by microorganism isolated from Brazilian soil contaminated with petroleum using polyethylene glycol (PEG) and potassium phosphate salt. At first step, the lipase was precipitated with ammonium sulfate, however only the protein contaminated was precipitated with 80% of saturation (purification factor – P/F=155.98 fold for aqueous phase). The enzyme, that remained at the aqueous solution was dialyzed against distilled water for 18h, the PF presented a decrease (32.17 fold). The enzymatic solution dialyzed was used to prepare an aqueous two-phase system (PEG/potassium phosphate). It was investigated different molecular weight of PEG to purify the lipase, and the best PF was obtained using PEG-8000 (123.89 fold), then it was studied the influence of PEG and potassium phosphate concentrations in the enzyme purification, the highest PF (123.89) was verified with 20% of PEG and 18% of potassium phosphate. It was added NaCl for increased the hydrophobicity between the phases, this addition increased the purification factor (141.65 fold). The pH value and temperature affected the partition of enzyme, the best purify condition was reached at pH 6.0 and 4°C (201,53 fold). According the results the best condition to purify the enzyme is PEG-8000/potassium phosphate (20/18%) with 6% of NaCl at pH 6.0 and 4°C.

Keywords lipolytic enzyme, purification, aqueous two-phase system

Optimisation of production of therapeutic peptides in Escherichia coli

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Biopharmaceuticals, biotechnologically produced therapeutic proteins (antibodies) and peptides, play an increasing role in pharmaceutical industry. They are used for the treatment of a wide range of human diseases such as autoimmune diseases, diabetes, anemia, disorders associated with lack of certain proteins (e.g. human growth hormone) and others.

For the expression and purification of pharmaceutically active peptides fused to maltose binding protein (MBP) and additionally carrying a 6 x His tag, a small (laboratory) scale procedure has been developed (see accompanying poster by Antosova et al.). This procedure enabled the production of small amounts of purified therapeutically active peptides and thus served as proof of principle. It is however not suited for the production of the (large) amounts needed for the production of drugs. The aims of this work was thus to develop a procedure that allows upscaling of expression/production and purification of peptide-fusion protein.

To identify suitable growth conditions for peptide fusion protein E. coli cultures were grown in a PC-controlled fermenter. First, different growth media were tested. Due to better aeration, cell densities in fermenter were ~ 1.5-fold as high as those obtained in Erlenmeyer flasks when standard complex (LB) growth medium was used in a batch procedure. By using a rich complex medium (TB) the obtained cell density could be increased additionally ~ 4-fold. However, it turned out that due to limitations in aeration the obtained yield was still lower than expected. To obtain higher cell densities, we thus switched to a fed-batch procedure in synthetic minimal medium. After the initial batch phase, growth was controlled by keeping aerobic conditions through controlling the supply of feeding solution in response to oxygen availability. This proceeding led to reduced growth rates, but to a significantly increased yield.

For the small (lab-scale) purification of peptide-fusion protein affinity chromatography using either batch procedures or gravity flow columns was carried out. It turned out that both, affinity chromatography using amylase resin, binding the MBP, or Ni⁺ resin, binding the 6 x His tag, allowed a one step purification to >90 % purity. However, whereas amylase resin is too expensive for production scale purification, the imidazole that is used to elute protein from Ni⁺ resin, lead to precipitation at higher protein concentrations. Thus, we developed a FPLC-based purification protocol consisting of an ion exchange chromatography (cation exchanger) step for pre-purification, the affinity chromatography step followed by a second ion-exchange column (anion exchanger) for removal of imidazole and buffer exchange. This protocol allowed the purification of peptide fusion protein without precipitation at a concentration of 0.7 mg/mL without precipitation.

In summary, we have successfully developed a scalable strategy for the production and purification of peptide-fusion proteins. Future work will be directed to the development of a most efficient procedure to separate the therapeutically active peptide from the fusion part and its final purification.

Keywords biopharmaceuticals; therapeutic peptide; bioreactor

Acknowledgement This work is supported by grant MPO 2A-2TP1/030.
Optimization of growth media for economical β-glucanase production by Bacillus sp.

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In nutrition of poultry, barley and wheat are commonly used for economical reasons but their efficiency as feed depends on various enzyme additives to better make use of the available nutrients. Beta-glucanase is one of these additives used for the complete utilization of feed component, β-glucan, found in barley and wheat. Unfortunately if beta-glucan remains undigested, it causes the viscosity of intestine to increase. In addition to its significance in animal breeding, especially for poultry and pigs, beta-glucanase enzyme has different roles in other fields. In plants, acting as an endocytic enzyme, β-1,3-1,4-glucan hydrolyses the β-1,3,1,4-glucan bond found in the cell wall of cereal endosperm. It is also involved in the utilization of glucose as an energy source for the young seedlings. In winemaking, it is used to improve the filterability of wine. In fungi, the enzyme is involved in biological processes such as cell expansion and cell-cell fusion. Microorganisms such as Bacillus subtilis, Bacillus macerans, Bacillus licheniformis, Clostridium thermocellum can produce high amounts of beta-glucanase. Catalytic features, stability, and activity under extreme conditions make beta-glucanases of microbial origin favorable for large scale production. The purpose of this study was optimize the economical production of a beta-glucanase enzyme from a newly isolated Bacillus strain to be used as an additive in poultry feed. Among the different carbon sources used (barley flour, wheat flour, and CMC), enzyme production with wheat flour was the highest. Then different organic (yeast extract and peptone) and inorganic (NaNO₃, (NH₄)SO₄) nitrogen sources were examined for enhanced beta-glucanase production. Higher enzyme yield was obtained with yeast extract as the nitrogen source whereas the Bacillus strain used in this work could not utilize inorganic nitrogen sources for beta-glucanase production. The best enzyme yield, was obtained when wheat was used as the carbon source and yeast extract was used as the nitrogen source.

Keyword: Bacillus sp., β-glucanase, β-glucan

Optimizing the production of biodiesel through the use of porc pancreatic lipase (PPL)

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Introduction and Experimental Procedures. Triglycerides (TG) of vegetable oils and fats are becoming increasingly important as alternative fuels for diesel engines due to the diminishing petroleum reserves [1]. However, their high viscosities and low volatilities do not permit their direct use either in oil/petrol blends or in any diesel engine type. Recently, several reports can be found on the production of biodiesel involving enzymatic catalytic protocols as greener alternatives [2]. We report here the preparation of a novel biodiesel by integrating glycerol into its structure via 1,3-selective ethanolysis using a pig pancreatic lipase (PPL). The ethanolysis reaction was performed in an stirred tank reactor (Batch), at atmospheric pressure and a temperature range from 40-60 °C, at pH = 11-13 and reaction times typically between 30 minutes and 24 hours. The effect of the temperature, cell/alcohol ratios and quantity of catalyst have also been investigated.

Results and discussion. Results have been summarised in Table 1. Quantitative conversions in the systems were found after 50 min. reaction, with increasing selectivities to FAEE (Fatty Acid Ethyl Ester). The formation of the maximum quantities of FAEE allowed by the action of the 1,3-stereoselective PPL were observed at very short times of reaction (5 min). With time, the DG are converted into MG until achieving the maximum MG content (33.3%).

Table 1. Composition, conversion and TOF, of the biofuel produced in the transesterification of 0,01 mol (12 mL) of sunflower oil, using 0,01 g of PPL (free form) and 6 mL of ethanol, at pH = 12 to 50 °C for one hour.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>FAEE %</th>
<th>MG+DG %</th>
<th>TG %</th>
<th>Conv. %</th>
<th>TOF (mmol/h gPPL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>66</td>
<td>16</td>
<td>17</td>
<td>83</td>
<td>7968</td>
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<tr>
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<td>70</td>
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<td>14</td>
<td>86</td>
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<td>88</td>
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<td>81</td>
<td>23</td>
<td>0</td>
<td>&gt;99</td>
<td>968</td>
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<tr>
<td>60</td>
<td>74</td>
<td>27</td>
<td>0</td>
<td>&gt;99</td>
<td>739</td>
</tr>
</tbody>
</table>

Interestingly, an increase in the quantity of PPL led to a decrease of the activity in the systems that might be related to the formation of agglomerates of enzymes, which prevented the access of the substrate to the active sites of the enzyme. A reduction in the conversion was also found with decreasing the alcohol/oil ratios in the reaction.

Conclusions. PPL can be used as biocatalyst to prepare a novel biofuel with composition and properties suitable to use in diesel engines, with the advantage of avoiding the generation of glycerol as byproduct in the process. This methodology can offer advantages compared to the conventional base catalysed process, in the search for new biofuels by incorporating glycerine that minimizes the waste production and improves the reaction conversion under greener conditions.

Acknowledgements

This research has been supported by the Instituto Andaluz de Biotecnología (Junta de Andalucía, Project BROANDALUS 08/1315/L5), Consejera de Educación y Ciencia de la Junta de Andalucía (FQM 0162), (FQM 0191), Ministerio de Educación y Ciencia Project CTQ 2007-65754-PPQ, Ministerio de Ciencia e Innovación CTQ 2008-01330/BQU).

References


Keywords: Pig Pancreatic Lipase (PPL); Biofuel; Ethanolysis; Fatty Acid Ethyl Ester (FAEE); Monoglyceride
Partial purification and characterization of five alpha amylases from a wheat local variety (Balady) during germination

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A local Saudi Arabian wheat (*Triticum aestivum*) variety (Balady) showed high levels of amylolytic activities at different stages of germination. The activity of α-amylase increased from day 0 to day 6 of germination, where it exhibited its highest level (2300 units/g seeds), followed by decrease of activity till day 16. Chromatography of 6 days old wheat seedlings of germination on DEAE–Sepharose column showed five forms of α-amylase activities (α-amylases AI, AII, AIII, AIV, and AV). The apparent K values of isoenzymes for hydrolyzing starch were 1.42 mg, 2.0 mg, 1.1 mg, 2.5 mg and 1.7 mg, respectively. α-Amylases AI, AII, AIII, AIV and AV were found to have sharp and broad pH optima of 5.5, 5.5-6.5, 5.0-6.0, 5.0-6.0 and 7.0, respectively. The temperature optima of wheat amylases are the same at 50°C. Thermal stability study showed that α-amylases AI, AIV and AV were stable up to 50°C after incubation for 15 min, while α-amylases AII and AIII were stable up to 40°C. The affinity between substrate and enzyme was detected only for glycogen and starch compared with other carbohydrates tested, where glycogen had more affinity than starch. Various metal ions such as Ca²⁺, Zn²⁺, Ni²⁺, Hg²⁺ and Cd²⁺ at 2 mM were tested for amylase activation/inhibition effect. Ca²⁺ is found to has activating effect as indicated by increased activity for all isoenzymes except of AII which is inhibited. In conclusion, these α-amylases from wheat have interesting characteristics such as low km value, broad pH optimum, high optimum temperature, high affinity toward starch and glycogen and activation by some metal as calcium. Therefore, these characterization meet the prerequisites need for food industry.

Phosphoglucomannase mutase GlmM of *Streptococcus pneumoniae* is activated by endogenous eukaryotic-type protein kinase StkP

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Phosphoglucomannase mutase (GlmM) is an essential enzyme catalyzing interconversion of glucosamine-6-phosphate and glucosamine-1-phosphate isomers, the first step in the biosynthetic pathway leading to the formation of UDP-N-acetyllglucosamine. To be active, this enzyme must be phosphorylated. The initial phosphorylation of purified *E. coli* GlmM is achieved in vitro during an autophosphorylation process. We recently showed that phosphoglucomannase mutase of *Streptococcus pneumoniae* is an endogenous substrate of eukaryotic-type serine/threonine protein kinase StkP. Therefore, we hypothesized, that phosphorylation of GlmM by protein kinase StkP in *S. pneumoniae* could be a factor regulating the activation of GlmM.

By using recombinant proteins in coupled enzymatic assay, we analyzed the effect of GlmM phosphorylation on its activity. Our results showed that the specific activity of GlmM reached the maximum after phosphorylation mediated by protein kinase StkP. Mutant protein was prepared by site directed mutagenesis and both the wild type and mutant forms were analyzed for their enzymatic activity. In contrast to the wild type protein, mutant form with Ser99 to Ala exchange showed no enzymatic activity.

In conclusion, our results showed that phosphorylation of GlmM by protein kinase StkP in *S. pneumoniae* could be a factor regulating the activation of GlmM and consequently the flow of metabolites in the cell wall biosynthetic pathways.
Production and characterization of thermostable phytase from Bacillus strain isolated from rhizosphere of Acacia cyanophylla Lindley

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Phytases (myo-inositol hexakisphosphate phosphohydrolase) are a special class of phosphatase that catalyze the sequential hydrolysis of phytates, the major storage form of phosphate in grains and oil seed, to myo-inositol pentakisphosphate (IP5) or to less phosphorylated myo-inositol phosphates (IPn) (Kerovuo, Rouvinen and Hatzack, 2000; Quan et al., 2004) or inorganic phosphate (IP) (Wyss et al., 1999). In recent years, phytases have been studied extensively, because of the great interest in its application as feed additive, processing of human food, synthesis of lower inositol phosphates, and ecological importance.

Bacillus phytases have considerable potential in commercial and environmental applications, because of their desirable activity profile under neutral pH, strict substrate specificity and higher thermal stability during animal feed-pelleting process in which temperature can reach to 80-100°C.

Isolate SDP1 from Bacillus genus, producing a thermostable phytase was isolated from the rhizosphere of Acacia cyanophylla Lindley from Cukurova region in Turkey. An extracellular phytase activity increased markedly in late stationary phase. The highest enzyme activity was observed after the 4th day of cultivation. Medium composition were optimized for an efficient phytase production. The crude phytase showed good activity at broad pH and temperature range. Almost ~10% and ~20% activity losses were observed on enzyme activity at 70°C and 80°C, respectively after 2 hours incubation at pH 7.0. According to our preliminary results, isolate SDP1, member of Bacillus subtilis group, can be proposed as a good phytase producer.

This research was supported by Marmara University (BAPKO) by the project number BAPKO FEN-C-DRP-171108-0267.

Keywords: Phytase, feed additive, myo-inositol, thermostable

Production of a new biodiesel by using a low cost lipase derived from Thermomyces lanuginosus and a response surface methodology

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Introduction. Biodiesel production has received considerable attention in the recent years as a biodegradable and non polluting fuel [1–4]. Utilizing soluble lipase biocatalyst presented an alternative approach to lipase-mediated biodiesel production. This new biodiesel integrates the glycerine as a monoglyceride, avoiding in this way its elimination. The multi factorial design of the response surface methodology (RSM) was employed to evaluate the effects on the conversion of oil into FAEE (Fatty Acid Ethyl Ester) and MG (Monoglycerides) of several conditions as temperature, molar ratio of ethanol to oil and pH. Water amount and concentration of lipase were also investigated. Soluble lipase Lipopan 50 BG, produced by submerged fermentation of genetically modified Thermomyces lanuginosus/Aspergillus oryzae microorganisms, was proposed here as a low cost biocatalyst for a new biodiesel production with sunflower oil. The results indicated that pH, molar ratio of ethanol to oil and water content were significant factors on the conversion.

Results and discussion. In Figure 1 it is shown the great influence of pH, and to lesser extent of the ratio oil / ethanol (proportion) in the conversion, while temperature did not affect significantly. In another graph it is shown the influence of the water content, which presents a maximum at 15 μL of added water.

Figure 1. Water content influence and Pareto graph.

Conclusions. Lipopan 50 BG can be used as biocatalyst in the production of a new kind of biodiesel that integrates glycerin as monoglycerides. It is possible to optimize conditions to prepare a new kind of biodiesel with composition and properties suitable to use in diesel engines. This method has the advantages of avoiding the generation of glycerol as byproduct in the process and its short reaction time. This process minimizes waste generation and maximizing efficiency of the process.

Acknowledgements. This research has been supported by Instituto Andaluz de Biotecnología (Junta de Andalucía, Project BIOANDALUS 08/15-L35), Consejería de Educación y Ciencia de la Junta de Andalucía (PQM 0162), (PQM 5191), Ministerio de Educación y Ciencia Project CTQ 2007-65754-PPQ, Ministerio de Ciencia e Innovación CTQ 2008-01330/BQU).

References

Keywords: Lipase; Thermomyces lanuginosus; Biofuel; Ethanolysis; Fatty Acid Ethyl Ester (FAEE); Monoglyceride
Production of Amylases, CMCases, Xylanases and Ligninolytic Enzymes by White-rot Fungi in Solid and Liquid Fermentation

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The most abundant polysaccharides in the environment are Cellulose and Hemicellulose as well as Lignin, present in the forestry by-products. The white-rot fungi are capable of transforming Lignin, present in the agroforestry waste, due to their ample enzymatic system made up of ligninolytic enzymes like Laccase and Manganese Peroxidase, as well as Endoglucanases and Xylanases. The degradation of such ligninocellulosic material has an enormous potential for the production of biofuels. For such reasons the objective of this work was to determine the capacity that the white-rot fungi, isolated from Nuevo Leon, Mexico, exhibit in the production of CMCases, Xylanases, Laccase and MnP in solid as well as in liquid fermentation, using wheat bran as substrate. Nineteen white-rot fungi were utilized. Sixteen of them isolated in Nuevo Leon, Mexico, and three reference strains, Pleurotus ostreatus (Po) ATCC 58053, Bjerkandera adusta (Ba) and Phanerochaete chrysosporium (Pch). For the fermentation in solid, 4 g wheat bran, saturated with 20 mL of salts (2 g KH₂PO₄, 0.5 g de MgSO₄·7H₂O, 0.5 g CaCl₂ y 0.5 g KCl, all per litre), was used. The fermentation in liquid was conducted in 250 mL Erlenmeyer flasks, with 90 mL of mineral solution and 45 g/L wheat bran, pH was adjusted to 5.0. Each flask was inoculated with three fragments of mycelium and incubated for ten days at 30 °C. The production of hydrolytic, ligninolytic and cellulolytic enzymes was greater in liquid fermentation than in solid, where amylase activity was detected in 14 of the 19 strains with Cu1 showing the greatest activity (16.52 U/g de substrate). In contrast, Trametes villosa presented an activity of 45.12 U/g of substrate in liquid media. The maximum CMCases activity obtained in solid media were 24.22 U/g (Cu1) followed by 17.37 U/g (P. chrysosporium), whereas in liquid media the greatest activity was present in Cu1 at 61.82 U/g. The activity of xylanases was detected in five strains under solid fermentation. Again, the maximum activity of xylanases in solid fermentation was detected in the strain Cu1 with 231 U/g of substrate. The results in liquid fermentation showed that Schizophyllum commune, S2, SL2 and Cu1 presented values of 533.22, 502, 484 and 404 U/g of substrate, respectively. The activity of MnP was detected in thirteen strains in solid fermentation and in fifteen strains in liquid. However the greatest activity was detected in CC1 with 5.3 U/g substrate in liquid fermentation. The previously given data contrasts with that determined by different researchers which indicates that solid fermentation considerably favors the production of ligninolytic, as well as cellulolytic enzymes. In the solid fermentation, CH7 presented the maximum value for Laccase, with 82.8 U/g substrate, while in Pch it was not detected. In Ba it was at 6 U/g substrate, and in Po it was at 25 U/g substrate. In liquid, by contrast, the highest activities were given by CH7 and CH8--- both strains belonging to the genus Pycnoporus---, with 221 and 227 U/g substrate, respectively. The results indicate that the amylase activity founded in the assays strains was the highest activity followed by CMCases and Xylanases. These native strains are potential candidates to future studies for their application in the production of fermentable sugars using agroforestry wastes. The obtained results in the present study indicate that the assayed fungi, primarily the native fungi, can be potential candidates for their application in the production of fermentable sugars without leaving out other areas of environmental interest. However, more specific studies, which will allow the optimization of the production of the studied enzymes in the present work, are necessary.

Keywords: Solid state fermentation, native fungi, ligninolytic enzymes.
Role of valine residue conserved at extra-sugar binding space in hydrolysis and transglycosylation activities of cyclomaltodextrinase-family enzymes


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Glycoside hydrolase family 13 (GH-13) is the largest family of α-glycosidases characterized to date and the members of this family display a variety of catalytic properties. Therefore, many GH-13 glycosidases have been studied to identify key residues required for their function and to determine their mechanisms of action.

Cyclomaltodextrins (CD)-degrading enzymes belong to GH-13, but they have unique catalytic characteristics compared to other GH-13 enzymes. There are three subclasses of CD-degrading enzymes: cycloextrinases (CDases; EC 3.2.1.54), maltogenic amylases (MAases; EC 3.2.1.133), and neopullulanases (NPases; EC 3.2.1.135). Based on their catalytic properties and structural similarities, these three subclasses can be reclassified into an unified class of CDase-family enzymes. Most enzymes in CDase-family are known to be capable of hydrolyzing various substrates such as cyclodextrins, starch, pullulan, and maltotriosaccharides. Especially, CDases and MAases can hydrolyze acarbose, a potent α-glucosidase inhibitor, to glucose and acarviosine-glucose (AG) and transfer AG to various sugar acceptors. Additionally, CDase-family enzymes can simultaneously catalyze both cleavage of α-(1,4)-glycosidic linkages and transglycosylation of the resulting products to C3-, C4-, or C6-hydroxyl groups of various acceptor sugar molecules.

Thermus MAase (ThMA; a typical MAase) mutant K-33, showing quite different acarbose hydrolysis pattern, has been found via combinatorial saturation mutagenesis of V329-A330-N331-E332 (VANE) residues placed in extra-sugar-binding space (ESBS). While ThMA wild-type (VANE) hydrolyzes acarbose to glucose and AG, ThMA K-33 (altered to SGDE) produced glucose, maltose, and acarviosine instead of AG. In addition, ThMA K-33 showed remarkable reduction of transglycosylation activity compared to ThMA wild-type. In order to examine the roles of each residue, V329-A330-N331 residues in ThMA were replaced with SAN, VGN, VAD, SGN, SAD, and VGD residues by site-directed mutagenesis. Substrate specificity and transferring activity of ThMA and its mutants were comparatively investigated. As a result, only ThMA mutants, containing SAN, SGN, and SAD residues, showed some acarbose hydrolysis patterns as ThMA mutant K-33, which means V329 residue can play important roles in acarbose hydrolysis of ThMA. Saturation mutagenesis of the valine residue substituted by the other amino acid residues confirmed that the valine 329 can be a key residue for the unique acarbose-hydrolyzing patterns found from ThMA mutants K-33.

In order to confirm these findings to be generalized in the other CDase-family enzymes, the corresponding residue of ThMA was replaced from Bacillus halodurans C-125 (BHC) or Listeria innocua CLIP11262 (LICD) with a serine residue by site-directed mutagenesis. Characterization of BHC V327S and LICD V340S verified that the corresponding substitution of valine residue to serine resulted in similar changes in acarbose hydrolysis pattern of each CDases. In addition to acarbose hydrolysis, the substrate specificity and transglycosylation activity of CDases and their mutants were comparatively analyzed. In this work, it has been suggested that substrate specificities, hydrolyzing activity, and transferring activity of CDase-family enzymes can be modulated by changing amino acid residues near ESBS, including V329 (in ThMA). These results will be applicable to develop novel carbohydrate-active enzymes engineered for the production of functional carbohydrate materials.

Keywords cyclomaltodextrinases; site-directed mutagenesis; conserved valine residue; acarbose hydrolysis; transglycosylation

Structural characterization of the propeptide NH₂-terminal of the precursor of pulmonary surfactant protein B (SP-B)

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Pulmonary surfactant is a complex mixture of lipids and proteins necessary to prevent the alveolar collapse during expiration. Among the hydrolitic proteins of the system surfactant protein B (SP-B) is produced from the proteolytic processing of a precursor along the secretory pathway in pneumocytes. The NH₂-terminal flanking propeptide (SP-Bₚ₃) is thought to act as chaperone of SP-B [1].

Previously, we have produced a recombinant form of SP-Bₚ₃ as fusion protein with MBP in Escherichia coli, processed the fusion protein by proteolytic cleavage with Factor Xa and purified SP-Bₚ₃ through ion-exchange chromatography followed by mass spectrometry and tryptic digestion characterization and amino terminal elucidation through Edman sequencing [2]. Also, we have studied the aggregation of SP-Bₚ₃ when the pH of the medium was acidified to mimic changes in pH occurring along the exocytic pathway, as well as the effect that stabilizers and crowding agents such as arginine and Ficoll 70 respectively have on the secondary and tertiary structure of the protein as it suffers acid aggregation [3].

The secondary structure of SP-Bₚ₃ is sensible to pH changes of the medium and we have studied the influence that the reversion of acid to neutral pH has on its circular dichroism signal as well as the effect of the ionic strength. Moreover, the structural stability of the protein to high temperature has also been analyzed by checking the dichroic signal of the protein at 20 °C after being heated up to 85 °C. The effect that urea and guanidinium chloride have on the structural stability of the protein has also been determined by means of circular dichroism and fluorescence spectroscopy measurements.

References


Structural characterization of Xylanase II from *Trichoderma reesei* QM9414

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Xylan is the major hemicellulase component in plant cell walls and its hydrolysis plays an important role in the breakdown process of plant material in nature [1]. The filamentous mesophilic fungus *Trichoderma reesei* (anamorph of the pantropical ascomycete *Hypocrea jecorina*) is known to produce several xylanases (EC 3.2.1.8, named XYNI, XYNII, XYNIII and XYNIV) which show differences in molecular weight, pI, and optimum pH [2] and hydrolyses the β-1, 4 bonds in the main xylan chain generating a mixture of xylo-oligosaccharides and a β-xylosidase (EC 3.2.1.37) which cleaves off the terminal xylose unit from the non-reducing end of xylo-oligosaccharides. Nowadays, the wide interest in xylan removal from food, feed, textile and pulp paper industries makes the study of xylan degrading enzymes stability a major goal in biotechnology [3].

Previously, we have grown *Trichoderma reesei* QM9414 on wheat straw as sole carbon source and we have optimized the conditions of the fungus growth and of cellulases and hemicellulases production. Besides our work with cellulases, we have purified two hemicellulases following ammonium sulphate precipitation, DEAE-sepharse CL-6B and Ultragel AcA 44 chromatographies. Moreover, we have characterized and determined the mechanism of catalysis of β-xylosidase. Also, we have immobilized β-xylosidase on nylon powder, designed a bioreactor to hold the immobilized enzyme and study its thermostability. Regarding enzymes with activity towards xylan, the alkaline XYNII has been purified and studies dealing with the thermal stability of XYNII and the protective role of polyhydroxyl cosolvents were carried out [4].

Now, we have studied the effect on XYNII of several compounds, including protectors and inhibitors by means of determining its enzymatic activity, thermostability and structure. Several techniques such as circular dichroism and fluorescence spectroscopy of intrinsic fluorophores have been employed to determine the Tm of the enzyme.

References

The ratio laccase/Mn peroxidase in solid state cultures of *Pleurotus pulmonarius* affects the ability to decolourize industrial dyes.

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White rot fungus (WRF) are the only microorganisms known to be able to degrade the highly recalcitrant natural polymer lignin because they possess a powerful enzymatic system formed mainly by peroxidases and laccases. Due to high inactivity of its oxidative enzymes, many studies have focused the WRF as useful to degrade dyes, suggesting the bioremediation as an environment-friendly and cost-competitive alternative for dyed wastewater treatment. The WRF *Pleurotus pulmonarius* is easily cultivated in various lignocellulosic substrates such as wood chips, corn wheat, rice straw, cotton stalks and waste hulls. More recently, several food wastes have been used as substrate mainly in solid state cultures of WRF. *P. pulmonarius* when cultured under submerged and solid state conditions using wheat bran as substrate produces high levels of laccase and very low levels of Mn peroxidase activity. For this reason, the capability of *P. pulmonarius* to decolourise textile dyes is associated with the laccase activity. More recently, however, it has been described that when *P. pulmonarius* was cultured in wheat bran solid state medium at low initial moisture content, it produced elevated amounts of both enzymes. Mn peroxidase and laccase. The objectives of this study were to compare the production of laccase and Mn peroxidase by *P. pulmonarius* in solid state cultures using three different substrates, wheat bran, corn cob and pineapple peel at different initial moisture contents and to evaluate the capability of these cultures to decolourize some industrial dyes.

The fungus was cultured on potato dextrose agar Petri dishes (PDA) for up to 2 weeks at 28 °C. When the Petri dish was fully covered with mycelia, mycelial plugs measuring 10 mm in diameter were made and used as inoculum. *P. pulmonarius* was cultivated under solid state conditions at 28 °C in the dark. Three disks with 10 mm of diameter from the growing edge of the mycelium on PDA plates were transferred to 250 ml Erlenmeyer flasks containing 5 g of each substrate at different initial moisture content. Five to 50 ml of distilled water was added to adjust the moisture content. To extract the enzymes, cold water was added to each culture flask and the mixtures were maintained in a incubator shaker at 4°C and 150 rpm for 2 h. To test the ability of cultures to decolourise industrial dyes (Remazol brilliant blue, RBBR; Ethy violet, Methylene blue, Poly R478 and congo red), each dye was membrane-filtered through a 0.45 μm cellulose nitrate filter and mixed with the corn cob medium, previously autoclaved, to a final concentration of 200 ppm. After 15 days, the residual dyes in the cultures were extracted firstly with 50 ml of water followed by 50 ml of a mixture of methanol:acetone:water (1:1:1). Dye disappearance was determined spectrophotometrically by monitoring the absorbance at the wavelength of maximum absorbance for each dye. In control cultures, either dye or the fungus (abiotic control) was omitted. To calculate the residual dye in the cultures, the total dye extracted with water and organic mixture in the abiotic control was considered as 100%. The amount of adsorbed dye on corn cob medium after growth of the fungus was always less than 10%. Laccase activity was determined with ABTS as the substrate. Oxidation of ABTS was monitored as absorbance increase at 420 nm. The Mn peroxidase activity was assayed by following the oxidation of MnO₄⁻ in malonate buffer in the presence of H₂O₂. Manganic ions form a complex with malonate, which absorbs at 270 nm. One unit (U) of enzymatic activity was defined as the amount of enzyme required to produce 1 μmol product per min and was expressed as U/L. Laccase was the main ligninolytic enzyme produced by the fungus in wheat bran cultures (maximum laccase activity of 2,800 U/L and Mn peroxidase inferior to 50 U/L) and its production was positively affected by increases in initial moisture content (IMC). In pineapple peel cultures both enzymes were produced at high amounts, being high initial moisture content (80-90%) the best condition to produce laccase (2,500 U/L) and low initial moisture content (70-75%), the best condition to produce Mn peroxidase (2,000 U/L). The substrate where the initial moisture content had the strongest effect in the production of enzymes was corn cob. Initial moisture content of 85-90% was the best condition to produce Mn peroxidase (2,000 U/L) and very low Mn peroxidase activity was detected in these filtrates (less than 5 U/L). The best initial moisture contents to produce Mn peroxidase changed from 50 to 65% (500 U/L), a condition where the production of laccase was very low (less than 20 U/L). The capability of fungus to decolourize industrial dyes was studied using corn cob cultures at 60 and 85% of initial moisture content. Both cultures efficiently decolourized RBBR and barely decolourised Poly R478. The cultures with 60% of initial moisture content were more efficient (p<0.05) in the dye decolourization of congo red, methylene blue and ethyl violet. In the present work, we showed that the cultivation of *P. pulmonarius* under solid state conditions using corn cob and pineapple peel as substrates resulted in a convenient condition to produce also high amounts of Mn peroxidase. By varying only the initial moisture content, the use of corn cob as substrate allowed the obtainment of cell free extracts rich either in laccase either in Mn peroxidase. Our data suggest that both enzymes laccase and Mn peroxidase from *P. pulmonarius* are equally efficient in decolorize RBBR. However, Mn peroxidase appear as the main responsible for the decolourisation of congo red, ethyl violet and methylene blue by *P. pulmonarius*.

Keywords: dye decolourisation; ligninolytic enzymes; *Pleurotus pulmonarius*; solid state cultures.
Utilization of agroindustrial residues as substrates for production of pectinolytic enzymes by biological agent “G088”.

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Recently, a great number of microorganisms, isolated from different materials have been screened, for their capabilities of degrading the polysaccharides present in the plant biomass producing pectinases (pectinolytic enzymes) in solid substrates. A number of agroindustrial residues and by-products such as orange bagasse, sugar cane bagasse, wheat meal, coffee hull and other food processing residues are effective substrates for the production of pectinolytic enzymes. The application of residues is a way to utilize alternative substrates and solving pollution problems for the industries and cost in enzyme production. Pectinolytic enzymes, which degrade the pectin present in the medium lamella and primary cell wall, have a great commercial importance for several industrial applications, such as to improve the juice yields and clarification in the industry of food, beer-making and pharmaceutical and textile industry. It was intended with this work to evaluate the potential of producing pectinolytic enzymes by biological agent “G088” in plant residues utilized as substrates. The first step was inoculating the biological agent in the different substrates: orange bagasse, sugar cane bagasse, grape skin, passion fruit skin, coffee and rice hull. The enzymatic activities of polygalacturonase (PG) and pectin methyl esterase (PME) of the substrates were evaluated; the best result for each enzyme was related with cropping time and type of substrate. Analyses of pectin quantification, pH, moisture and titrable acidity of the substrates with inoculum and of the centesimal composition of the growth of plant pathogens such as Pantoea agglomerans, and several Pseudomonas syringae pv. glycinea (Psg), as well as the opportunistic fungal pathogen Candida albicans. Since the mode of action of Pa48b against plant and human pathogens, is elusive, we looked for the molecular basis for the biocontrol potential of Pa48b.

Recently, a great number of microorganisms, isolated from different materials have been screened, for their capabilities of degrading the polysaccharides present in the plant biomass producing pectinases (pectinolytic enzymes) in solid substrates. A number of agroindustrial residues and by-products such as orange bagasse, sugar cane bagasse, wheat meal, coffee hull and other food processing residues are effective substrates for the production of pectinolytic enzymes. The application of residues is a way to utilize alternative substrates and solving pollution problems for the industries and cost in enzyme production. Pectinolytic enzymes, which degrade the pectin present in the medium lamella and primary cell wall, have a great commercial importance for several industrial applications, such as to improve the juice yields and clarification in the industry of food, beer-making and pharmaceutical and textile industry. It was intended with this work to evaluate the potential of producing pectinolytic enzymes by biological agent “G088” in plant residues utilized as substrates. The first step was in inoculating the biological agent in the different substrates: orange bagasse, sugar cane bagasse, grape skin, passion fruit skin, coffee and rice hull. The enzymatic activities of polygalacturonase (PG) and pectin methyl esterase (PME) of the substrates were evaluated; the best result for each enzyme was related with cropping time and type of substrate. Analyses of pectin quantification, pH, moisture and titrable acidity of the substrates with inoculum and of the centesimal composition of the substrates without any inoculum were done. The different substrates showed activity of the pectinases, polygalacturonase (PG) and pectin methyl esterase (PME), standing out grape skin and rice hull. But, the best substrate for production of PG (117.35 U/ g) and PME (1760 U/g) at 14 days was grape skin. The composition of the substrate has a direct influence on the production of both PG and PME.

Keywords: pectinametilesterase – polygalacturonase - solid substrate.

A novel epoxide antibiotic isolated from Pantoea agglomerans 48b/90 inhibits economically important plant pathogens and the human pathogen Candida albicans

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Microbial pathogens pose a major threat to many plants and can cause enormous losses in agriculture. Microorganisms that antagonize pathogens can offer a way to fight plant diseases that is more environmentally friendly than chemical treatment; such diseases include fire blight, which is caused by Erwinia amylovora and affects many roaceous plants, e.g. apple and pear.

Suitable strains for biocontrol agents are often plant-associated microorganisms that are forced under natural conditions to defend their ecological niches and thus adapted to compete with plant pathogens. The species Pantoea agglomerans (formerly Erwinia herbicola) comprises many strains that are promising sources for biocontrol agents. P. agglomerans are ubiquitous in nature, inhabiting plant surfaces, water, soil, animals and humans. Several Pantoea isolates are known to efficiently inhibit E. amylovora in planta. In vitro experiments have revealed some antibiotics from P. agglomerans and uncovered how they act against E. amylovora. The known antibiotics produced by P. agglomerans strains, which belong to diverse chemical classes and affect different molecular targets, exhibit both narrow and broad spectrum activity.

P. agglomerans 48b/90 (Pa48b), an epiphyte from soybean leaves, attracted our attention because it strongly inhibits the growth of plant pathogens E. amylovora and Pseudomonas syringae pv. glycinea (Psg), as well as the opportunistic fungal pathogen Candida albicans. Since the mode of action of Pa48b against plant and human pathogens, is elusive, we looked for the molecular basis for the biocontrol potential of Pa48b.

The epiphyte Pa48b has been isolated from soybean leaves and found to be well adapted to its niche. Pa48b produces an antibiotic with broad activity against Gram-negative bacteria e.g. Erwinia amylovora, Agrobacterium tumefaciens, Escherichia coli, several Pseudomonas syringae pathovars, Serratia marcescens, the Gram-positive Bacillus subtilis and the yeasts Candida albicans and Yarrowia lipolytica. Consequently, Pa48b is a promising biocontrol agent against various microbial plant diseases and offering possibilities of therapeutic intervention directed against Candida albicans, the causative agent of invasive mycoses that increased significantly over the past two decades.

In order to characterize the compound with high activity against plant pathogens and Candida albicans, a bioassay-guided isolation approach was used. A highly polar antibiotic was obtained after anion exchange chromatography and HILIC-HPLC purification. The purified antibiotic turned out to be stable at extreme pH; in addition, it was resistant to heat and treatment with proteinase K and β-lactamase. Its formation is associated with growth and it is temperature dependent: its rate of production is optimal between 8°C and 12°C.

Using HR-ESI-MS and NMR experiments, the structure of the compound was identified as 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine (I). This compound has already been isolated by Shoji et al. (1989) from Serratia plymuthica CB-25. However, I has been neither isolated from P. agglomerans nor characterized as highly active against plant pathogens such as E. amylovora and P. syringae pathovars.

In contrast to the impact of many antibiotics from P. agglomerans such as pantocin A and B or herbicolin O, the impact of 2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine cannot be compensated for by supplementing the medium with amino acids or casein hydrolysate. Therefore I is different than most other antibiotics from P. agglomerans strains.

Keywords: Pantoea agglomerans, antibiotic, Candida albicans, biocontrol, plant pathogens
Amine Derivatives of Fungal Monascus Pigment Inhibiting the Adipogenesis of 3T3-L1 Cells

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We have reported that amino acid derivatives of monascus pigment have antimicrobial, cholesterol-lowering, and lipase-inhibitory activities. Still, it remains a challenge to create various derivatives with biological activities. In this study, after orange monascus pigment was produced by fungal Monascus cultivation in a 5 L jar fermenter, various derivatives were synthesized by reaction of the pigment with forty-seven amines. These compounds were tested to have an inhibitory activity for the differentiation of preadipocyte 3T3-L1 cells to adipocyte cells. Among them, nine compounds showed significant activities and three derivatives of (R)-(+)-1-(1-naphtyl) ethylamine, 2-(p-toyly)-ethylamine, and 4-phenylbutylamine having exhibited high activities were chosen for next experiments. The preadipocyte 3T3-L1 cells were cultivated in the differentiation medium containing a pigment derivative for 6 days. Then, the cells were stained with Olig red O to identify exogenous or endogenous lipid deposits. The addition of amine derivatives apparently reduced the lipid content of cells in a dose-dependent manner, resulting in decrease of triglyceride (TG) droplets as well as inhibition of TG formation. The derivatives of 4-phenylbutylamine and 2-(p-toyly)-ethylamine showed inhibitory activities of 41.5% at 10 μM and 46.2% at 25 μM, respectively. The derivatives down-regulated the transcription factors PPARγ and C/EBPα, which are known to be strongly related to adipocyte differentiation. The derivatives significantly reduced expression of the transcription factors and inhibited normal development from preadipocytes to adipocytes. The derivative-associated attenuation of PPARγ and C/EBPα was accompanied by a decrease in abundance of the proteins. These results indicate that the derivatives can inhibit the expression/activation steps of PPARγ and C/EBPα, resulting in blocking of adipogenesis.

Keywords monascus pigment; fungal cultivation; 3T3-L1 cell; adipogenesis

Bacillus cereus hemolysin II and its various applications

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One of several cytolytic proteins produced by opportunistic pathogen B. cereus is hemolysin II (HlyII). The hlyII gene is widely spread among bacteria, which belong to the B. cereus group, including B. thuringiensis, some subspecies of which are used as insecticidal; and B. anthracis is an agent of anthrax, a mortal disease of man and animals. HlyII is a secreted microbial protein, which belong to the oligomeric β-barrel pore-forming toxins family that includes the Staphylococcus aureus α-toxin. HlyII can disrupt membranes of erythrocytes and other eukaryotic cells in vitro by forming membrane ionic oligomeric pores. Pore formation leads to perturbation of cell ion–osmotic homeostasis and to cell death and lysis. The hlyII expression in B. subtilis renders bacteria hemolytic. Expression of HlyII (recombinant B. subtilis::hlyII) in the crustacean gut leads to destruction of intestine cells, followed by a gradual disruption of other tissues. Fluorescent microscopy reveals post-infection changes in mitochondrial potential of intestine tissues during the first 24 h of infection, suggesting that formation of ionic pores leads to cell lysis and finally, to the host death. The hemolytic activity of hlyII-encoded B. subtilis strains in culture medium are positively correlated with virulence in D. magna. Expression of hlyII in B. subtilis::hlyII::hlyIIR is strongly suppressed by HlyIIR regulator and this recombinant is not pathogenic to the crustacean. HlyII expression and pathogenicity of bacteria is controlled by a negative transcriptional regulator. Hemolysin II can lead to death of macroorganisms. So, the search of drug that inhibited the activity of HlyII (for example cycloheximide) is needed. The data presented show that hemolysin II, when acts alone, is sufficient virulence factor, and the regulation of hlyII expression could be an important step in the adaptation of bacteria to different environmental niches. Potential applications of protein pores in medical biotechnology are discussed.
Bacteriocin Production by Bean Root Bacteria

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Bacteriocin production of native isolates of Rhizobium was investigation. Six isolates of bacteriocin producing Rhizobium bacteria were isolated from bean root nodules in Turkey. Isolate Y39 characterized in this study was one of the most effective isolates. The possible bacteriocin from produced by Rhizobium spp. isolate Y39 was inhibitory to a broad range of indicator microorganisms, including Micrococcus sp., Bacillus isolates CB and CB1, Actinomycetes isolates A1 and A5, Azotobacter spp. isolates C12 and C35, Pseudomonas phaseolicola, Xanthomonas phaseolicola, Fusarium culmorum, P. moniliforme, P. solani, F. oxysporum, Cladosporium herbale and Rhizoctonia solani. Bacteriocin was stable at 60 °C but the activity was lost when the temperature used at bacteriocins. It was resistant to RN/Aase and lysosome but sensitive to proteinase K and trypsin. Some of properties of the compound showed suggest a proteinaceous nature.

Keywords : Rhizobium isolates, bacteriocin, antimicrobial activity

Biopolymerization of Carvacrol and Optimization of Reaction Conditions Through Surface Response Methodology (RSM)*

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In recent decades, the enzyme-catalyzed polymerization, shortly enzymatic polymerization or biopolymerization, has been of increasing importance as a new trend in macromolecular science. Enzyme catalysis has provided new synthetic strategy for useful polymers, most of which are difficult to produce by conventional chemical catalysts. In enzymatic polymerization, the polymer can be obtained under mild reaction conditions without using toxic reagents. Therefore, the enzymatic polymerization has a large potential as an environmental-friendly synthetic process of polymeric materials, providing a good example to achieve “green polymer chemistry.”

The enzymatic synthesis of phenolic polymers has been extensively investigated due to their special features such as antioxidant, analgesic, fragrance and flavour and usage in food, cosmetics and pharmaceutical industries at large. Phenolic terpenes, which are ingredients of many essential oils found in plants used as flavouring and fragrance agents in perfumery, cosmetics, food and pharmaceutical industries. The concept of forming more functional structures upon biopolymerization with unusual properties experienced in other phenolic substances can lead to the opportunity of creating novel film forming agents, stabilizers, coagulants, dispersants, antioxidants, glues, binders, deodorizers, flavours and fragrances, agrochemicals, antimicrobials, wound healing preparations, insecticides, pesticides, drugs or drug carriers, transdermal preparations, as flavour and aroma binding, slow-release agents in cosmetics and foods, bio-nano-technological products or productions for electrical as well as electronic applications.

In this respect, carvacrol as the main constituent of oregano oil, which is an important essential oil exported from Turkey was selected as the model phenolic terpene and the laccase was employed as the model enzyme in this study. The study involved the realization of the biopolymerization of carvacrol monomer, the determination of the optimum reaction conditions in order to establish the maximum yield, statistical investigation/assessment of the effects of operational conditions such as solvent type and concentration, monomer concentration, enzyme activity and pH on the product amount and yield.

All biopolymerization experiments were conducted batchwise in a closed, temperature controlled system containing solvent, monomer, enzyme and the buffer for pH control. 14 Different polar and nonpolar solvents were attempted to synthesize the carvacrol biopolymers. As a result, overall 93% (w/w) polymer yield was obtained by using ethanol as a solvent under optimized conditions.

Batch experiments with four reactions parameters were planned by using Design Expert 6.0 (trial version) programme. The intervals of reaction parameters were selected as 3-8 units for pH, 0.1-7U/mL for enzyme activity concentration, 0.5-65 g/L for carvacrol concentration and 75 45-90 (v/v) for ethanol concentration, respectively. Maximum polymer amount was attained with 45% (v/v) ethanol -sodium acetate buffer at pH 5.5, 25°C, 3.55 U/mL enzyme and 32.75 g/L initial carvacrol concentrations.

Chemical structure of the carvacrol biopolymer was characterized by using spectroscopic techniques such as ¹H- and ¹³C-NMR, FT-IR and UV. Although the carvacrol monomer is soluble in all organic solvents except partially in water, the carvacrol biopolymer - a white powder- is water insoluble but soluble in organic solvents such as diethyl ether, chloroform and dichloromethane.

Furthermore the biological properties of the biopolymer was evaluated in various bioassays. It was determined that the carvacrol polymer showed good antimicrobial and antioxidant properties same as carvacrol by using in vitro microdilution assays. By using the in vivo Chorioallantoic membrane assay, it was found that both carvacrol and its biopolymer were non-toxic and non-irritant at the tested concentration of 50 mg/mL.

The results of this study proved that the laccase catalyzed carvacrol provided the formation of a novel product with biological activities.

Keywords : Enzyme catalysis, Biopolymerization, Terpenes, Laccase, Carvacrol

* This work was financially supported by TÜBİTAK 108M015 project.
**Biosurfactant Production by Candida pelliculosa and C. sphaerica Isolated from Soil Contaminated with Lead**

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Surfactants are amphiphatic molecules, which contain a hydrophobic and a hydrophilic group. Due to the presence of these two groups in the same molecule, surfactants can decrease surface and interfacial tension in water/hydrocarbon systems. The surfactants in current use are chemically derived from petroleum, however, interest in microbial surfactants has been steadily increasing, as surface-active biomolecules that are produced by a variety of microorganisms, have gained importance in the fields of enhanced oil recovery, environmental bioremediation, food processing and pharmaceuticals owing to their unique properties such as higher biodegradability and lower toxicity. The aim of this work was isolation and identification of yeasts from contaminated soil with lead from Belo Jardim, Pernambuco State, Brazil. The potential of biosurfactant production was investigated. The results indicated the presence of *Candida torpicalis* and *C. sphaerica*. The biosurfactant production was carried out evaluating the emulsification activity using low cost regional substrate babasu oil, as carbon source. The cultures were grown by the continuous batch process. The strains inocula size were also evaluated. The strains were grown in the presence of babasu oil and D-glucose, and inocula of 1% and 5% showed higher emulsification activity during the stationary growth phase, corresponding to 96% of cultivation, and the highest biopolymer production was occurred. In that period of growth was observed highest cell concentrations. The biochemical analysis revealed that the purified biopolymer was constituted by high amount of protein, and low content of lipids and carbohydrates, respectively. Qualitative enzymatic assays demonstrated that *C. tropicalis* exhibited esterase production, and *C. sphaerica* exhibited esterase and urease activities. The results indicated that *C. sphaerica* isolated from lead polluted soil demonstrated a high potential of the emulsifier activity, and suggesting that the presence of lead is not a limitation factor for biosurfactant production.

**Keywords:** Biosurfactant, Candida sphaerica, emulsifier activity, Lead

Supported by CNPq, CAPES, UPE, and UNICAP.

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**Biosurfactant production by Chromobacterium violaceum using alternative sources: corn steep liquor (industrial waste), lactose and corn oil**

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Interest in microbial surfactants has increased considerably in recent years, especially due to their potential of application. Recently, research related to optimizing the production of biosurfactants from substrates and regional sources demonstrated the production of those compounds. The biosurfactants have been tested in many environmental applications, such as in bioremediation, dispersion of oily stains and oil recovery, replacing the chemical surfactants. It also can be used in food, cosmetics, and detergents, and agricultural. These properties make surfactants suitable for a wide range of industrial applications involving: detergency, emulsification, lubrication, foaming capacity, wetting, solubilizing and dispersion phases. The vast majority of commercially available surfactant is synthesized from petroleum products. However, increasing environmental awareness among consumers, combined with new legislation to control the environment led to the search for natural surfactants as alternatives to existing products. This paper aims to biosurfactant production by *Chromobacterium violaceum* –Violsun, using industrial waste as well as evaluating the surface tension, index and activity of emulsification. The surface tension by *C. violaceum* showed values between 28 and 40 mN/m. However, the best result was observed in the assay 8 and 12 used in the factorial design carried out with greater amounts of corn oil, reducing the water tension of 71 mN/m to 28.98 mN/m. The n-hexadecane emulsion also formed in all conditions ranging between 0.9 and 6.5 UAE. These results indicate that the *C. violaceum* has higher potential for biosurfactant production.

**Keywords:** Chromobacterium violaceum, alternative sources, biosurfactant

Research supported by CAPES, CNPq and FINEP.
Biosynthesis of amino acids sulfur in *Saccharomyces cerevisiae* is affected by fermentation conditions in beer production

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Beer elaboration process is still considered, at least in part, an art. It is well known that both yeast and wort play an important role in the quality of final product, because several wort-compounds have influence in aroma and taste. In response to wort composition, the metabolic pathways can be modified in the yeast, and final product could result with high concentration of undesirable volatile sulfur compounds (VSC), mainly H₂S and SO₂. In this work we analyzed the expression of genes involved in the biosynthesis of VSC in *Saccharomyces cerevisiae* growing under different fermentation conditions. We designed two kinds of wort: one produced with high content of malt (above 50%) and other with low content of malt (below 50%), and were inoculated with yeast previously maintained at 4°C and 18°C, respectively. Fermentations were conducted at 16°C for seven days. In order to evaluate genetic expression, the yeast was collected at second day and gene expression analysis was done using DNA microarrays. Results demonstrated that the wort composition plays an important role in the biosynthesis of sulfur amino acids and it could impact in the generation of VSC.

**Keywords:** *Saccharomyces cerevisiae*, MET2, MET3, Microarray, VSC

Characterisation of Polyhydroxyalkanoate Produced by Haloarchaea Isolated from Salt pans of Goa-India.

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Several halophilic Archaea, isolated from salt pans of Goa – India produced polymer in nutrient rich medium with 25% crude salt and or in mineral salts medium with 20% NaCl, at pH 7, ambient temperature (23-28°C), which were detectable by Nile blue staining. Accumulation of polymer in cells was in the order of genus *Haloferax* > *Halococcus* > *Halobacterium* > *Haloarcula*. Further, the amount of polymer produced by individual isolate of a genus varied with: the type of media used; sugars, acids and natural product of agriculture used, as sole source of carbon; condition of growth, as batch or in fermentor system. For production and isolation of polymer, the individual isolates were grown in mineral salts medium with 2% starch without any growth factors. *Haloferax volcanii* (GUFLF 7), *Haloarcula* (ATCC BAA 652, GUBF9), and *Halobacterium* strain R1 (MTCC 3265, GUSF) gave 12%, 7.5% and 6% polymer per gram cell wet weight. Yield per cent of polymer was twice more in cells grown as batch cultures than in cells grown in 5.0L stirred fermentor a further increase in accumulation of 0.75 times was noted with increase of growth temperature to 42°C. The accumulated polymer was extractable in chloroform and or by simple, rapid, physico-chemical-lysis method, developed in laboratory. Physicochemical and thermal analysis of polymer, isolated from GUSF, GUBF9, GUBF19, GUBF1 and GUF7 revealed the polyhydroxyalkanoate nature of the polymer.

The presentation will discuss these and other findings related to the biodegradability of the polymer produced and the potential of Haloarchaea for production of biodegradable bio-plastics from microbes.

**Key words:** PHA, Polyhydroxyalkanoate, Haloarchaea, Biopolymers, Biodegradation.
Characterization of marine isolates with extracellular protease production and their commercial application

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Protease has an immense application in industrial and commercial field, keeping it in mind, we choose marine coastal ecosystem (support ~3.67x 10³⁰ microorganisms) to isolate protease producing bacteria. Seven extracellular protease producing pure bacteria were isolated from the two sites of the marine coast of West Bengal; Digha and Mandarmani and one site of Andhra Pradesh; Vijay. It was characterized morphologically, physiologically and at the biochemical level. Molecular characterization was done by 16s rDNA based sequence analysis. All were observed to be Gram positive bacilli. It was further confirmed by Real time PCR analysis. Presence of endospore in all indicated their survivability under stressful condition. Except one of the bacterium, all of them showed presence of catalase and oxidase. The isolates were considered to be non-pathogenic, as they either showed negative result to lecithinase, or did not grow in the medium. These results were reconfirmed through Real time PCR pathogenicity detection test from ABH. Optimum pH and temperature range were pH 6 -12 and 20°C-40°C reflecting their range of adaptability. Jaggery and tamarind were proved to be better carbon source for all, than other prescribed carbon sources. Tolerance of higher concentration of heavy metal salts namely Al, Fe, Ni, Pb, Zn, could make them potent bioremedial candidate to clean the environment. Extracellular protease secreted by all of the pure isolates reflect more or less same efficiency to destain the cloth Beside the protease, two of the strains were also found to produce extracellular lipase.

Keywords: marine; protease; bioremedial; degumming; additive to detergent.

Chitin and chitosan produced by Mucoralean fungi using a new economic medium- Corn Steep

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Chitin and chitosan hold a great economic value as due to their versatile biological activities and chemical applications, mainly in medical. Recent advances in fermentation technologies suggest that the cultivation of selected fungi can provide an alternative source of chitin and chitosan. The amount of these polysaccharides depends of the fungi species and culture conditions. Filamentous fungi have been considered an attractive source of chitin and chitosan for industrial applications because their specific products can be manufactured under standardized conditions. However, to optimize the production of chitin and chitosan from fungi, it's usually used complex or synthetics cultures media, which are expensive. It’s becomes necessary to obtain economic culture media that promote the growth of fungi and stimulate the production of the polymers. Ecomomic microbial culture media normally use vegetables components. Microbiological process were studied for production of chitin and chitosan by Mucoralean fungi grown in a new economic medium, Corn Steep. A laboratory assay were carried out to evaluate the Cunninghamella elegans, Rhizopus arrhizus, Absidia corymbifera and Mucor javanicus growth using Corn Steep medium during 96 hours, under agitation (125 rpm) at 28°C. The mycelial biomass were determined following lyophilization. Among all of the strain two of them have amylase activity, which help them to use as a additive with detergent as they showed better efficiency to destain the cloth. Beside the protease, two of the strains were also found to produce extracellular lipase.

A higher production of biomass, statistically significant (p = 0.0018) can be verified to M. javanicus with average dry weight corresponding to 4.22g/L in 96 hours of growth. On the other hand A. corymbifera produced lower yields of biomass (1.98g/L). There was no statistical difference between R. arrhizus (3.6g/L) and C. elegans (3.2g/L) for biomass production. In addition, the best yield of chitosan (5.0mg/g) are obtained to A. corymbifera, R. arrhizus and C. elegans. M. javanicus showed lower production of chitosan (4.2mg/g). The higher yield of chitin was verified to A. corymbifera (297.9mg/g), with statistically significant (p=0.004). No difference was observed between C. elegans and M. javanicus chitin production (184mg/g). R. arrhizus showed lower chitin yield (146mg/g). Chitin and chitosan showed degree of deactelation and viscosimetric molecular weight in a range of 70-89% and 2.59- 3.25 x 10⁴ g/mol, respectively. The results showed a great potential of biomass of mucoralean fungi grown in the new economic medium, Corn Steep, with important biotechnological application. Our results suggest that Corn Steep medium improves chitin and chitosan production and the mycelial biomass of mucoralean fungi may be used as an alternative source of these polymers.

Keywords: Corn Steep, biopolymers, polysaccharides
Compared production of lutein-enriched biomass from a new strain of microalgae in different photobioreactors

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Microalgal biomass has gained importance due to the commercial interest of its applications, ranging from vitamins, pigments or biofuel production to CO\textsubscript{2} fixation. In order to reduce biomass production costs, it is necessary to optimize the microalgal biomass production process, using efficient photobioreactors and optimizing process engineering parameters, as well as identifying new strains of rapid growth and commercial interest of further applications.

In the present work, a compared study of the productivity of the microalgae \textit{Chlamydomonas acidophila}, a new strain isolated by our group from the Tinto river (an acidic river with extreme conditions for life in the southwest of Spain) what has been found to be promising for the industrial production of high commercial value antioxidants as lutein, was carried out. Microalgal growth and productivity of lutein-enriched biomass was assessed in three types of 5 liters photobioreactors: fluidized-bed column, tubular reactor and flat panel reactor.

The results of preliminary experiments show the 1.5cm light path flat panel photobioreactor, to be the most efficient culture system, with top growth rates of 0.8d\textsuperscript{-1} and volumetric biomass productivities of 0.3 g \textit{l}\textsuperscript{-1}d\textsuperscript{-1}, with the highest lutein content published up to now.

Acknowledgements: This work was supported by grant AGL2006-12741 (Ministerio de Ciencia e Innovación, España), grant AGR-4337 (Junta de Andalucía, España) and grant BIOANDALUS 08/14/3.6 (Universidad de Málaga y Consejería de Innovación, Ciencia y Empresa, España).

Keywords: Microalgae, photobioreactor, biomass.

Development of a carob based medium for mannitol production by \textit{Leuconostoc fructosum} NRRL B-2041

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Mannitol obtained by microbial fermentation was approved by FDA in 2004 and its production at industrial scale has already been implemented. Microbial production of mannitol currently competes with the chemical process as it presents numerous advantages over the chemical hydrogenation and still has great improvement possibilities. The medium formulation cost is a significant contributor to the overall mannitol production cost \cite{1} and therefore the evaluation of alternative economic feedstocks for carbon sources and low-cost supplements are currently a major concern.

Carob (\textit{Ceratonia siliqua}) is a Mediterranean perennial tree producing pods that contain seeds (10%) and sugar-rich pulp (90%), which exhibits higher sugar content than sugar cane. The pulp is currently an under-utilized by-product of the locust bean gum production, as its main application is as animal feed.

\textit{Leuconostoc fructosum} NRRL B-2041 has been found to be a mannitol overproducer when grown in supplemented carob syrup based medium \cite{2}. In order to minimize media formulation costs, the composition of carob based culture medium was evaluated using a Plackett-Burman statistical design. The studied factors were the type of hydrolysis (acid/enzymatic), hydrolysate concentration (carob syrup volume:supplements volume, 1:1/3:1), and supplementation (beef extract, corn steep liquor - CSL, peptone and yeast extract). The studied variables impact differently on bioprocess performance. Supplementation negatively affected products yields, but increases productivities. Specifically, mannitol production was positively influenced by beef extract. This supplement also positively affected both lactic acid and glycerol production. The later was also positively affected by peptone supplementation and by enzymatic hydrolysate type. Beef extract together with the low-cost CSL, strongly influenced substrate uptake. On the light of these results, yeast extract and peptone can possibly be omitted leading to considerable savings in medium costs. Beef extract and CSL were identified as the most relevant supplements that should be further studied.

It was also demonstrated that the hydrolysate concentration and hydrolysis type might also have a role on bioprocess efficiency, as it significantly affected products profile. On the best conditions \textit{Lc. fructosum} NRRL B-2041 can produce 42 g/l of mannitol which corresponds to a volumetric productivity of 1.75 g/lh and a yield on fructose of 1.48 g/g.

The prospects and drawbacks arising from the use of a carob syrup based medium for mannitol production at industrial scale are presented and discussed.

Keywords: Carob, \textit{Leuconostoc fructosum}, mannitol, Plackett-Burman statistical design, supplementation

\begin{thebibliography}{9}
\bibitem{2} Moniz, P. \textit{et al.} Physiological characterization of mannitol overproducing strains in carob based medium. This meeting.
\end{thebibliography}

This work was supported by AdI, Project ValorAlfa (70/00326). ARS Culture Collection (National Centre for Agricultural Utilization Research, Peoria, IL, USA) is gratefully acknowledged for supplying \textit{Leuconostoc fructosum}.
Effect of culture conditions on the production of an extracellular protease by a Bacillus sp isolated from soil samples of Tehran park

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Proteases are one of the most important industrial enzymes and used in a variety of industrial applications, such as laundry detergents, pharmaceutical industry, leather industry in dehairing and bating of hides, manufacture of protein hydrolyzates, food industry, silver recovery from X-ray films, and even in waste processing industry. These enzymes account for about 60% of the total enzyme market.

Soil samples of Tehran parks were screened for proteolytic Bacilli. Among eighteen protease producers one of the isolates obtained from north east of Tehran, was selected for further experimental studies. This isolate was identified as Bacillus subtilis isolate D13AO7 based on partial sequencing of 16S rRNA. Various nutritional and environmental parameters affected protease production by this Bacillus. Protease production by Bacillus subtilis isolate D13AO7 cultivated in liquid cultures containing 1% starch as a carbon source and 0.4% corn steep liquor as a nitrogen source reached a maximum at 24 h, with levels of 340.908 U/mL. Starch and maltose were the best substrates for enzyme production while some pure sugars such as fructose, glucose and sucrose could not influence production of protease. Among various organic nitrogen sources corn steep liquor, which is commercial, was found as a best substrate followed by yeast extract, whey protein and beef extract. Bacillus subtilis isolate D13AO7 could not utilize urea as an inorganic nitrogen source to grow and produce protease. The optimal pH and optimal temperature of enzyme production were 8.0 and 45°C, respectively. Studies on enzymatic characterization revealed that crude protease showed maximum activity at pH 9.0 and 60°C, which it is indicating the enzyme to be thermo-alkaline protease. These properties indicate the potential use of this bacterium and its protease for various industrial applications.
Enhanced growth and lipid anabolism in iron exposed cultures of *Chlamydomonas acidophila* isolated from an acidic environment.

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Tinto River, in the southwest region of Spain, is one of the most extensive examples of extreme acidic environments, exhibiting very low pH (ranging from 0.8 to 2.5) buffered by ferric iron, which dissolves other cationic metals including Zn, Mg, Al, As, and some anions as sulphate, up to high concentrations. Photosynthetic microalgae as *Chlamydomonas acidophila*, natural inhabitant of this environment, has adapted to acidic stress by expressing metal tolerance mechanisms. However, little is known about biochemical changes induced in *C. acidophila* by exposure to sublethal heavy metal concentrations. That information should be highly helpful to assessing the use of *C. acidophila* in both biomass production and metal removal processes. The aim of this work was to assess the effect of Fe3+, the most abundant metal ion in Tinto River, on growth and biochemical profile (protein, carbohydrate and lipid contents) of *C. acidophila*.

The presence of Fe3+ enhanced the growth of *C. acidophila* significantly. Iron concentration critically influences cell growth of *C. acidophila*, which may account for 35% higher than iron-free microalgal cultures in terms of cell density. Maximum growth rate obtained for *C. acidophila* cultivated in iron-added culture medium hardly differs from those of non-extremophile microalgal, making *C. acidophila* suitable for microalgal biomass outdoor production process in selective acidic culture medium. Accumulation of carbohydrates and decrease in protein content at increasing iron concentrations were also observed in our results. Furthermore, Fe3+ redirected carbon metabolism toward lipid accumulation as observed in terms of total lipid content which might be a useful tool for production of lipid enriched microalgal biomass.

Acknowledgements: This work has been supported by the Ministerio de Educación y Cultura (Grant AGL2006-12741), Junta de Andalucía (Proyectos de Excelencia, AGR-4337) and Universidad de Málaga and Consejería de Innovación, Ciencia y Empresa (Grant BIDANDALUS 08/14/L3.6).

Keywords: extremophiles, *Chlamydomonas acidophila*, iron, biochemical composition

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Extraction of clavulanic acid in aqueous two-phase systems followed by separation through ultrafiltration

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Clavulanic acid (CA) is an important beta-lactam antibiotic that acts as an inhibitor of beta-lactamase enzymes produced by pathogenic bacteria. The CA is produced by strains of *Streptomyces clavuligerus* by submerged and aerated crops of *S. clavuligerus*. In the extraction step have been studied processes with ultrafiltration membranes, and developed extraction processes through organic solvent and aqueous two-phase systems (ATPS), while the purification has been studied by a process involving adsorption by ion exchange. The ATPS, usually composed by polyethylene glycol (PEG) and a salt (eg phosphate) are used in the most of biotechnological process. This work aims to: 1) Get the extraction and ultrafiltration parameters of PEG/CA system, such as partition coefficient of CA (K_P) and resistivity of layer gel (I), for PEG’s of several molecular weights; 2) Evaluate the ultrafiltration performance on PEG rejection (σ_{PEG}) with and without ethanol. It is hoped with this work assess the feasibility of implementing the proposed process which will contribute to the development of a CA continuous process extraction.

It was found that the partition coefficient of clavulanic acid was around 4, while with the use of PEG-400 rose to 20, indicating good extraction. The results showed that the PEG rejection in ultrafiltration ranged between 13 and 84%, and the solutions PEG/CA containing ethanol had a lower rejection when compared with the initial solutions. High rejections of PEG indicate that a good part of this one has been retained by the ultrafiltration membrane, very interesting fact for subsequent purification processes in which the polymer is the main contaminant. Moreover, the resistivity values of the gel layer were little influenced by the molecular weight of PEG used, showing that the main agents responsible for the gel layer in the ultrafiltration are macromolecules, such as proteins.

Keywords: membrane; gel layer; resistivity; partition coefficient

Acknowledgements: This work has been supported by the Ministerio de Educación y Cultura (Grant AGL2006-12741), Junta de Andalucía (Proyectos de Excelencia, AGR-4337) and Universidad de Málaga and Consejería de Innovación, Ciencia y Empresa (Grant BIDANDALUS 08/14/L3.6).
Fermentation of grape marc for production of bioactive phenolic compounds

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Agro-industrial by-products are important sources of remarkable bioactive phenolic compounds, these compounds are of extreme relevance for food and pharmacological industries due to their great variety of biological activities. Fermentation of grape marc (Vitis vinifera) represents an environmentally clean technology for the production and extraction of bioactive phenolic compounds, providing high quality and high activity extracts.

In this work, the importance and benefits of solid state fermentation (SSF) is presented, pointing out this bioprocess as an alternative technology for the use of agro-industrial residues as substrates to produce valuable secondary metabolites. Physicochemical characterization of grape marc was carried out to assess its properties as support for SSF. Furthermore, eight different fungal strains were tested in order to evaluate their capacity to release phenolic compounds and invasiveness on grape marc.

In addition, High Performance Liquid Chromatography was employed for phenolic compounds determination. Results showed that grape marc has the qualities to be a good support for SSF. Members of Aspergillus group had higher invasive capacity than Penicillium strains on grape marc. A. niger PSH, A. niger PSH and A. niger Aa 20 increased the level of galic acid present in grape marc after 12h of culture of which A. niger PSH released the highest amount of pyrocatechol at 48h.

Keywords: grape marc, solid state fermentation, bioactive phenolic compounds

Green microalgae: source for healthy foods, novel biofuels and CO2 abatement

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Microalgae are definitely in the way to become a sustainable, easily reproducible natural green source for highly demanded market products. Within the current most outstanding applications of microalgae, the following can be cited: (1) production of functional foods, enriched in healthy bioactive molecules; (2) novel fuels, produced from microalgal fatty acids and (3) greenhouse gas abatement (CO2 mitigation), based on high cell density microalgal cultures technology. Besides, other traditional microalgal applications are currently increasing in competitiveness in a growing market demand for natural products, in which microalgae are widely recognized as a healthy, sustainable and biological renewable resource. Among these traditional applications, microalgae are being used (4) to improve the nutritional quality of animal feed, specially in aquaculture, (5) to produce enhanced fertilizers -in combination with other active components-, (6) to remove heavy metals from waste effluents and (7) to produce stable isotope biomolecules, very useful in metabolic engineering studies. Many of the relevant microalgae for the above applications can grow in extreme environments which at least meet any of those conditions considered not suitable for life: among them, high concentration of salt and/or metals, high temperature, high radiation and high/low pH. Moreover, the biosynthesis of many of the microalgal bioactive molecules of commercial importance takes place in conditions under which life is hardly possible. This work reviews recent advances in the field of biotechnological production of highly market demanded microalgal products.

Acknowledgements: This work has been supported by the Ministerio de Educación y Cultura (Projecto AGL2006-12741) and Junta de Andalucía (Proyecto de Excelencia, AGR-4337).

Keywords: microalgae, healthy foods, biofuels, CO2 abatement, microalgal biotechnology, market.
Growing kinetics of antimicrobial activity of *Streptomyces tubercidicus* brute extracts

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*Streptomyces* spp. is studied in function of its antibiotic production with industrial and pharmaceutical applications (Challis, 2008). The irregular use of antibiotics can lead to the development of resistant strains and the search for new antimicrobials is interesting (Jain & Jain, 2007) and encouraged. This work aims to evaluate the growing kinetics of *S. tubercidicus* and compares the data with its bioactivity against *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 25922.

*S. tubercidicus* was cultivated in ISP2 broth and incubated in 28°C with agitation (250 g). Microbial quantification, was done utilizing decimal dilutions in saline solution. A volume of 100 μL of each dilution were seeded on ISP2 agar and plates were incubated for 28°C for 48 to 72 h. Quantifications were done in triplicate and results expressed as log UFC mL⁻¹. The antimicrobial activity was done on 4 ml of the media to obtain the crude extract. The culture was centrifuged (15000 g/15 min in 20°C). Supernatant was filtered in 0,22 um membrane. 100 μL aliquots of crude extract were tested as doubling dilution. The crude extract was tested in a well diffusion assay in Müller-Hinton previously seeded with indicator microorganisms (10⁶ cells/mL). Plates were incubated at 37°C overnight in order to see the inhibition halos. Bioassays were evaluated in triplicate. Antimicrobial activities were expressed in arbitrary units/mL (UA/mL⁻¹).

Growing kinetics of *S. tubercidicus* was evaluated during 21 days. The major quantification was 7.88 log UFC mL⁻¹ seen the second day of incubation. The bioactive substances production was seen at the end of the exponential phase and the beginning of the stationary phase of growth (day two).

The fresh crude extract of *S. tubercidicus* showed a maximum antimicrobial potential of 200 UA mL⁻¹ against *Escherichia coli* and *Staphylococcus aureus*. The crude extract was active during 21 days, without alteration of the antimicrobial potential. The crude extract revealed three compounds (Figure 1) using Thin Layer Chromatography and UV illumination. Two compounds are less polar (seen with 254 nm) and the third is more polar (365 nm). The extract presented polar amino acids and one compound with minor oxygenated function (254 nm). Data presented here are important and the isolated microorganism is a valuable source of bioactive compounds against important Public Health-relevant microorganisms.

**Figure 1:** Crude extract of *Streptomyces tubercidicus* with different compounds revealed by Thin Layer Chromatography

**Keywords** *Streptomyces tubercidicus*; antimicrobial activity; growing kinetics; brute extract; thin layer chromatography
Identification and Emulsification Properties of a Biosurfactant Produced by Bacteria from Soil, Chiang Mai, Thailand

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Biosurfactants are valuable microbial amphiphilic molecules with effective surface-active and biological properties. One hundred and ninety-seven bacterial soil strains were screened for extracellular biosurfactant activity by an oil spreading technique. It was found that bacterial isolate SCMU 106 was capable of excellent biosurfactant production. Analysis of the morphological and biochemical characteristics revealed that this isolate is a member of the genus Pseudomonas. Phylogenetic analysis using 16S rDNA indicated that the isolated SCMU 106, at 99% similarity, is closely related to Pseudomonas aeruginosa. We measured the emulsification properties of the biosurfactant in terms of emulsification index (E24) and emulsion stability at high temperature. It was found that kerosene, at about 60%, was the best substrate when treated at 100 °C for 90 minutes. The biosurfactant will be further investigated for potential use in health and cosmetics applications.

Keywords biosurfactant, emulsification index, Pseudomonas aeruginosa

Isolation and antimicrobial activity of Streptomyces tubercidicus against pathogenic bacteria and fungi

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Streptomyces spp. produces many secondary metabolites, including antibiotics. The substances formation is coupled with the onset of development of the microorganism and the search of new substances is important. The aim of this work was to evaluate the bioactivity of Streptomyces tubercidicus isolated from Solanum lycocarpum St. Hill, a typical Brazilian tropical savanna tree in order to test its inhibitory capability against pathogenic bacteria and fungi. Typical colonies were purified in ISP2A slants and incubated 28 oC/10 days. Selected colonies were evaluated regarding its morphological, cultural and biochemical properties. The endophytic microorganism was identified using rRNA 16S. S. tubercidicus was cultivated in ISP2A and submitted to antibiosis test. After growing, microorganisms were inactivated with chloroform. The indicator microorganisms utilized were S. aureus (ATCC 29213), P. aeruginosa (ATCC 27853), E. coli (ATCC 25922) and C. albicans (ATCC 10231). The strains were reactivated in BHI broth and after 24 h, 200 μL of the cultures were put in tubes with 10 mL semi solid BHI. Tubes were shaken and solution was deposited in plates surface with the inactivated microorganism. Plates were incubated at 37º C/48 h to verify the occurrence of halos. S. tubercidicus presented inhibition against S. aureus and E. coli (Figure 1), and the substances are being purified using Sep Pak C18 chromatography. The strain had the potential to inhibit C. albicans with halos de 1,94 cm, but failed to inhibit P. aeruginosa. These results allowed us to devise and consider the isolated microorganism as a valuable source to the discovery of new bioactive compounds against important Public Health microorganisms.

S. tubercidicus presented inhibition against S. aureus and E. coli (Figure 1), and the substances are being purified using Sep Pak C18 chromatography. The strain had the potential to inhibit C. albicans with halos de 1,94 cm, but failed to inhibit P. aeruginosa. These results allowed us to devise and consider the isolated microorganism as a valuable source to the discovery of new bioactive compounds against important Public Health microorganisms.

Figure 1. Brut extract of Streptomyces tubercidicus and bioactivity using SepPak C18 chromatography

Key words: Streptomyces tubercidicus; antimicrobial activity; pathogenic bacteria; pathogenic fungi
Isolation, phenogenotypic identification and bioactivity of endophytic microorganisms intrinsically associated with Miconia albicans in Brazilian tropical savannah tree in Sao Carlos - SP

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Endophytic microorganisms live in symbiotic association with plants, have unique biological niche, and are potential producers of natural bioactive substances. This study aimed to isolate and characterize endophytic microorganisms from Miconia albicans from Brazilian tropical savannah of Sao Carlos - SP. Samples were characterized phenotypically from typical features of colony morphology and macroscopic measurements, physiological, biochemical and morpho-dyeing and genetically by 16S rRNA analysis. We evaluated the antagonistic potential of the isolates against Staphylococcus aureus ATCC 25923, Escherichia coli ATCC 25922, Serratia marcescens IAL 1475, Enterovococcus fecalis ATCC 29212, Shigella sonnet ATCC 10231 and Candida albicans ATCC 10231. Two samples (1 and 3) showed (Table 1) bioactivity against E. faecalis, with inhibition halos of 1.30 and 1.90 cm in diameter, respectively. A single sample (2) showed activity against S. marcescens (3.60 cm) with no significant bioactivity against the other microorganisms tested. Sample (4) showed activity against four of the six microorganisms tested: E. faecalis (3.15 cm), S. aureus (3.50 cm), C. albicans (3.35 cm) and E. coli (3.20 cm). In another sample (6) was detected bioactivity against E. faecalis and S. aureus, with inhibition zones of 1.80 and 1.50 cm, respectively. One of the samples (8) showed bioactivity against the greatest number of indicator microorganisms with inhibitory halos of 2.60 cm against E. faecalis, 1.50 cm against S. aureus, 2.40 cm against C. albicans, 2.10 cm against E. coli and 2.50 cm against S. sonnet. In two samples (5 and 7) there was no evidence of the bioactive ability against indicator microorganisms tested. Phenotypic analysis of 8 endophytic microorganisms isolated allowed us to classify 50% of these (samples 4, 6, 7 and 8) in the Actinomycetes group and the others were classified in the genus Streptomyces (samples 1, 2, 3 and 5). In the genotypic analysis of two isolates with bioactivity were identified as Nocardiosis Dassencolle (sample 3) and Amycolatopsis orientalis (sample 8), reporting for the first time its association with endophytic aerial parts of Miconia albicans from Brazilian tropical savannah in Sao Carlos, SP.

<table>
<thead>
<tr>
<th>Samples</th>
<th>E. faecalis</th>
<th>S. aureus</th>
<th>S. marcescens</th>
<th>C. albicans</th>
<th>E. coli</th>
<th>S. sonnet</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>1.30</td>
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<tr>
<td>3</td>
<td>1.90</td>
<td></td>
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<td></td>
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<tr>
<td>4</td>
<td>3.15</td>
<td>3.50</td>
<td></td>
<td>3.35</td>
<td>3.20</td>
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<td>5</td>
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<tr>
<td>6</td>
<td>1.30</td>
<td>1.50</td>
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<td>7</td>
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<tr>
<td>8</td>
<td>2.60</td>
<td>1.50</td>
<td></td>
<td>2.40</td>
<td>2.10</td>
<td>2.50</td>
</tr>
</tbody>
</table>

Table 1: Antibiosis obtained with endophytic microorganisms isolated from M. albicans against indicators microorganism

Keywords endophytic microorganisms, Miconia albicans, Brazilian tropical savannah, bioactivity

Phosphate solubilization of low solubility sources in liquid medium by an isolate of Aspergillus niger

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Brazil is a major producer of grains with an annual four million tonnes of phosphate fertilizers consumption which 50% are imported. Essential to plants, phosphorus is considered to be nutrient limiting crop production and is in limited availability in tropical soils. So high doses of phosphate fertilizers are need for the crops to obtain good productivity. Should be considered, therefore, that fertilization is one of the most pressing production costs by reducing the profit margin received by producers. Many microorganisms solubilize different forms of inorganic phosphates. The inoculation of phosphate solubilizing micro-organisms or the management of their populations have been suggested as a way to replace or reduce the use of soluble phosphorus fertilizers, through better use of existing or phosphates added to the soil and formed by the application of soluble fertilizers. The objective of this study was to evaluate the efficiency of the M22 isolate the fungus Aspergillus niger in phosphate solubilization in liquid medium.

The assessment of capacity for phosphate solubilization in liquid medium by the strain of Aspergillus M22, was held at the Microbiology Laboratory of EPAMIG the Federal University of Lavras-MG, Brazil. The Araxá phosphate (14 g kg–1) was inoculated with biomass of 1 mL (8 CFU mL–1) and so we incubated them at 28 °C, under agitation at 190 rpm for eight days. The efficiency of solubilization was determined and it was evaluated the pH and P content at the end of the incubation period. The data were submitted to analysis of variance and means compared by Tukey test at 5% probability, using the program Sisvar. In liquid medium, the isolate of Aspergillus niger showed higher amount of soluble P in Araxá phosphate than with the residue of phosphate rock. The isolate M22, on average, increased about four times the amount of soluble P in the middle with Araxá phosphate, compared to control, and about three times that of soluble P in the middle with the residue of phosphate rock. Isolate M22 demonstrated the ability to solubilize phosphates. Mechanisms in plants and microorganisms can be low-cost technologies and appropriate to stimulate the solubilization and increase the agronomic effectiveness of phosphate rock.

Table 1: Antibiogram from endophytic microorganisms isolated from M. albicans against indicators microorganism

Keywords endophytic microorganisms, Miconia albicans, Brazilian tropical savannah, bioactivity
Polyhydroxybutyrate production from cheese whey by recombinant E. coli

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Cheese whey containing 4.5% of lactose is the main by-product of dairy industry and represents an important environmental problem due to the large volumes produced and its high organic matter content [1]. Polyhydroxyalkanoates (PHA) can replace petroleum-derived synthetic plastics because of their similar material properties and complete biodegradability after disposal. The major problems in commercializing microbial PHA are related to the high costs of carbon source and downstream process involved. Only a few microorganisms are able to convert lactose into PHA, e.g. recombinant Escherichia coli strains harbouring PHA biosynthesis genes. This recombinant microorganism offers several advantages: high cell densities and polymers cell contents. (near 90% of total cell dry weight); ability to utilize several inexpensive carbon sources; relatively easy downstream processing of PHAs and lack of intracellular depolymerases [2].

Recombinant Escherichia coli strain MG1655 was modified through the inclusion of p(3HB)-synthesis genes of Cupriavidus necator into the chromosome of each bacterial strain. Seed cultures were prepared in a 500-ml flask containing 100 ml of mineral medium supplemented with whey [3]. Fed-batch cultivation, with the selected strain was carried out at 37°C in a 2 l fermentor. Feeding mode was pulse-wise, (20 g/l) or continuous (30 g/l/h). The oxygen supply and carbon feeding strategies influence on growth and storage was studied. Preliminary results showed that the recombinant E. coli strain CML3-1 was able to accumulate the highest P(3HB) content (49% of cell dry weight). This strain was tested in a fed batch reactor with whey supply feeding pulses and continuously. The best result achieved was 138 g/l of cell dry weight and 63% of P(3HB) content (Table 1).

The carbon feeding strategy, in test A and B, was coupled to the pH control [3], with whey addition when pH increased above 7.2, however when the pH reached that value the lactose was already zero. The genes of PHB-synthesis became inactive when lactose reached zero, what damaged the PHB productivity. This is the reason that the feeding strategy implemented by Lee [3] can not be applied with this strain.

<table>
<thead>
<tr>
<th>Test</th>
<th>Feeding</th>
<th>Aeration</th>
<th>CDW (g/l)</th>
<th>%PHB</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Pulses when pH reaches 7.2</td>
<td>O2 30% → O2 10% when OD = 60</td>
<td>99</td>
<td>45</td>
</tr>
<tr>
<td>B</td>
<td>Pulses when [lactose] reaches 0.5</td>
<td>O2 60% → O2 20% when OD = 100</td>
<td>138</td>
<td>20</td>
</tr>
<tr>
<td>C</td>
<td>Continuous feeding</td>
<td>O2 60%</td>
<td>32</td>
<td>63</td>
</tr>
</tbody>
</table>

In tests A and B, the oxygen limitation, after growth phase, caused a deviation of carbon to the anaerobic fermentation, resulting in organic acids production. The %PHB of test B was lower than in test A; the limitation 20% of O2 seemed to be not as efficient as the limitation of 10% applied in test A. The feeding strategy used in test C was inhibitory for cellular growth but the PHB content reached 63%. The high lactose concentration privileged the PHB-synthesis. The low value of CDW, related with the high pyruvate production detected, probably resulted from the high lactose concentration. In test C, cell disruption was observed, which could be a consequence of high polymer content, high external solute concentration or internal metabolites accumulation. The cell membrane disruption, at the end of the process, is extremely advantageous for the downstream processing, since the extraction of polymer from cells is the most expensive step of the process. The experimental results showed that is possible to use whey as a carbon source, with a microbial strain showing high storage content, high cell density and allowing for a simple downstream processing.


Keywords Polyhydroxybutyrate; cheese whey; recombinant Escherichia coli

Prevention of L-tyrosine by-production and improved performance of an Escherichia coli L-phenylalanine-producing strain using tyrA ssrA-like tagged alleles

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Currently, the construction of L-Phe producers usually begins from tyrosine-auxotrophic mutants containing a disruption of the tyrA gene, which codes for chorismate mutase (CM, EC 5.4.99.5)/prephenate dehydratase (PDH, EC 1.3.1.12) (reviewed by Sprenger (2007)). This is done to prevent branching of the chorismate, the common precursor of the aromatic biosynthetic pathway, and the accumulation of L-Tyr as a by-product, the removal of which creates problems in downstream processing. Conversely, application of Tyr-auxotroph mutants requires the addition of L-Tyr to a fermentation medium, thereby increasing the cost of L-Phe. We constructed an L-Phe-producing strain that does not require L-Tyr for growth and does not allow L-Tyr accumulation as a byproduct in an S-3 fermentor.

To choose the appropriate level of TyrA activity, we varied the sensitivity of TyrA to proteosynthesis using ssrA-mediated tagging (Keller et al., 1998). The ssrA tag, an 11-aa peptide (AANDENYALAA) added to the C-termminus of proteins that is stalled during translation, targets proteins for degradation by cytoplasmic proteases ClpXP and ClpAP (Flynn et al., 2001). To weaken the binding of ClpX, we modified residues 9-11 using the custom tag AANDENYALDD (A-LDD). To weaken the binding of ClpA, residue 1 of the custom tag was deleted, resulting in ANDEYALDD (LDD) and ANDEYALAA (LAA). The ssrA and the designed tags were integrated toward the 3’ end of tyrA into the chromosome of E. coli BW25113 by in vivo Red-dependent recombination (Dansken & Wanner, 2000), with the simultaneous untagging of the nearest pheA-gene.

Strains BW25113ΔphecA with different tyrA-tag alleles and their isogenic variant BW25113ΔphecA tyrA-wt were tested for growth in M9 medium supplemented with L-Phe. According to their growth rates (μ), the strains were placed in an order that could be predicted according to the sensitivity of ssrA-like tags to proteolysis. BW25113ΔphecA tyrA-LDD and BW25113ΔphecA tyrA-A-LDD had nearly the same growth rate as BW25113ΔphecA tyrA-ssrA (μ=0.4-0.5 h -1). For BW25113ΔphecA tyrA-LAA, μ was decreased (0.35 h -1), and BW25113ΔphecA tyrA-ssrA had the lowest growth rate (μ = 0.1-0.2 h -1). The growth of the latter strain was fully restored by addition of L-Tyr (50 μg/ml) into the medium.

However, PDH enzymatic activity for the modified products of the tyrA-gene measured in crude extracts of growing cells did not correlate with the growth rate. Only for BW25113ΔphecA tyrA-LDD, PDH activities were practically the same in probes from cells harvested from the logarithmic phase of growth, and the enzymes demonstrated the same sensitivity to feedback inhibition by L-Tyr. In the stationary phase, PDH activity decreased 3- and 5-fold for BW25113ΔphecA tyrA-LDD, respectively. It appears likely that tag-modified TyrA became more sensitive to proteolysis activated in the stationary phase as compared to the native analog. The PDH activities of crude extracts of other strains were significantly decreased, likely due to intense degradation of TyrA-A-LDD, TyrA-LAA and TyrA-ssrA under the conditions employed.

To examine the influence of tyrA-tag genes on L-Phe and L-Tyr accumulation, these genes were introduced into E. coli L-Phe-producing strain DW269 (Imazumi et al., 2007). DW269 is a derivative of MG1655, harboring mutated araG and phcE encoding DAHP-synthase and CM/prephenate dehydratase, respectively, which are insensitive to feedback inhibition by L-Phe. This strain contained tyrA, which was substituted by different tyrA-tag alleles.

The obtained strains were tested in batch fermentors (BIOSTAT Q with a working volume of 0.3 l, BBI, Germany) on the glucose-containing medium. The medium was supplemented with L-Tyr for DW269. As the initial amount of L-Tyr in the medium determined the total biomass accumulation for DW269, the introduction tyrA-wt, tyrA-LAA and tyrA-A-LDD encoding rather stable TyrA into DY69 increased biomass accumulation of the corresponding strains by ~1.3-1.5-fold and accordingly decreased L-Phe production by 1.3-1.7-fold. In contrast, strains coding unstable TyrA-LAA or TyrA-ssrA accumulated nearly the same or lower biomass than DY69 and did not significantly decrease Phe production. All strains containing tyrA-tag produced lower levels of L-Tyr (by more than 10-fold) compared to DW269ΔphecA tyrA-wt. For DW269ΔphecA tyrA-wt and BW25113ΔphecA tyrA-LDD, the TyrA/w ratio was less than 0.04%.
A novel approach that included the engineering of controllable protein degradation was therefore successfully applied to the improvement of an amino acid-producing strain.

**Keywords** L-Phe production, tyrA-tag, tyrosine prototrophic strain

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**Production of mycotoxins Zearalenones and Aflatoxins by selected strains of *Fusarium* sp. and *Aspergillus flavus***

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In this work it will be discussed some important aspects and experiments involving HPLC-MS and HPLC-MS/MS for monitoring both simple and complex fungal metabolites and other natural products as well. The main focus will be in the usage of MS/MS experiments (CID-MS) and other specific experiments, such as Selected Reaction Monitoring (SRM), during mycotoxins production. Mycotoxins are secondary metabolites produced by fungi which are capable of causing disease and death in humans and other animals. Because of their pharmacological activity, some mycotoxins and many of their derivatives have been using as antibiotics, growth promoters, and other kinds of drugs for many years. Herein, we report the production of Zearalenones and Aflatoxins, two important type of active mycotoxins by means of fungal fermentation technology. Both compounds were obtained using solid state fermentation (SSF) and submerged fermentation (SmF) from selected strains of *Fusarium* sp. and *Aspergillus flavus*. Using SSF it was possible to reach production levels over than 51.1 mg/Kg. Currently, we are improving our production capability in order to raise up such levels since the production of high-quality mycotoxins is a key issue for industrial and academic-related fields.

**Keywords** Zearalenones; Aflatoxins; *Fusarium* sp.; *Aspergillus flavus*; HPLC-MS/MS
Production of lipids in different operational conditions by several marine and freshwater microalgae

M. Vila, E. Diaz, M. de la Vega, A. Simaitie and R. León

Microalgae are able to harvest solar energy and convert it into chemical-bond energy with high efficiency thanks to the photosynthetic process. The potential of this ability for sequestration of undesirable CO2 emissions and to produce high added value lipids is being widely studied, but to achieve outdoor large scale economically feasible production of microalgal lipids a careful selection of microalgal strains is necessary. The chosen strains have to grow at high rate over a wide range of temperatures and irradiances and accumulate a high percentage of lipids to ensure high lipids productivities at the local climatological conditions. We were interested in two classes of lipids, the carotenoids, lycopophilic terpenoids involved in light-harvesting and photoprotection against photooxidative damage, and the triacyl glycerides. Commercial carotenoids production is particularly interesting due to their antioxidant, colorant, provitamin and therapeutic abilities. While Microalgal TAGs, which after trans-esterification in the presence of methanol can be transformed into methyl esters of the corresponding fatty acids, are receiving increasing attention due to their potential as a source of biodiesel.

After a preliminary screening, two saline and two freshwater new microalgae strains were isolated from the Odiel river marshlands sited in Huelva (Southwest Spain) and their growth rate and their ability to accumulate lipids in response to different environmental conditions were studied. First we determined the growth rate of the new isolated strains at extreme temperature and light regimes to find the periods of the year at which the open door culture of these strains could be carried out with optimal growth rate. The quantity and quality of the lipids accumulated by the microalgae at different light intensities, temperatures, and concentrations of nitrogen source was analyzed. We found specific growth rates ranging between 0.02 and 0.5 h\(^{-1}\) and lipid percentages between 18% and 55%. In general those conditions unfavorable for growth were observed to stimulate the accumulation of total lipids.

Keywords: lipids, microalgae, stress conditions;

Acknowledgements: We thank the Spanish Ministry of Education for financial support (AGL2007-65303-C02-01)

Production of micro-organisms and metabolites for the food industry and agribusiness in a bio-factory in Brazil

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1 Agricultural and Livestock Research Institution of Minas Gerais State – Brazil
2 Federal University of Lavras, Minas Gerais State – Brazil

The great biodiversity turns Brazil in an open laboratory however it hasn’t been very much studied. A bio-factory with a technological base was born after obtaining positive results during twenty years of researches developed by its creators. It develops products from selected microorganisms that are able to promote yield protection, pest and diseases control, water purification and the improvement of phosphate fertilizer process solubilization, among others. The bio-factory came in like a small company, supported in its origin by Lavras Federal University Innovation Nucleus (NINTEC-UFLA), but with a great grown potential based in its affinity with the world demands by sustainables yield systems, safe products, under the food safe and environmental protection view points. The bio-factory has as mission to supply food industries and agribusiness with products and services of high quality aiming to be the first producers of innovative inputs in Brazil. The bio-factory sector has maintained a constant responsible for the collection, identification and assessment of micro-organisms and their capacity for direct use or its metabolites in different processes. The selected microorganisms are properly preserved until they are used.
Productivity of *Chlorella sorokiniana* in a short light-path (SLP) panel photobioreactor under high irradiance

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Maximal productivity of a 14 mm light-path panel photobioreactor under high irradiance was determined. Under continuous illumination of 2100 μmol photons m⁻² s⁻¹ with red LEDs (light emitting diodes) the effect of dilution rate on photobioreactor productivity was studied. The light intensity used in this work is similar to the maximal irradiance on a horizontal surface at latitudes lower than 37º.

*Chlorella sorokiniana*, a fast-growing green microalgae, was used as a reference strain in this study. The dilution rate was varied from 0.06 h⁻¹ to 0.26 h⁻¹. The maximal productivity was reached at a dilution rate of 0.24 h⁻¹, with a value of 7.7 g of dry weight m⁻² h⁻¹ and a volumetric productivity of 0.5 g of dry weight L⁻¹ h⁻¹. At this dilution rate the biomass concentration inside the reactor was 2.1 g L⁻¹ and the photosynthetic efficiency was 1.0 g dry weight per mol photons. This biomass yield on light energy is high but still lower than the theoretical maximal yield of 1.8 g mol photons⁻¹ which must be related to photosaturation and thermal dissipation of absorbed light energy.

**Key words:** panel photobioreactor, high irradiance, productivity, photosynthetic efficiency, *Chlorella sorokiniana*.

**Acknowledgements:** This work was financially supported by the University of Huelva and MEC (Grant AGL2006-12741) in Spain, and a SenterNovem subsidy (the Netherlands) in the frame of the “Unieke Kans Regeling” program, grant number 02013 with Technogrow BV as industrial partner.

Screening of Algerian lactic acid bacteria on their antilisterial activity

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Forty six samples from dairy and faecal origins were analysed for the presence of bacteriocin-producing lactic acid bacteria by the spot on lawn test. From these samples, thirty eight strains of lactic acid bacteria (LAB) were isolated with fifteen strains able to inhibit the growth of *Listeria monocytogenes*. When these isolates were evaluated by the well diffusion assay, five of these strains produced a proteinaceous substances active against *L. monocytogenes* EGDs. These isolates were identified phenotypically and genotypically by sequencing the gene coding the 16S rRNA. Two isolates were identified respectively as *Lactobacillus paracasei* subsp. *paracasei* and *Lactococcus lactis* subsp. *lactis*; the others are belonging to *Enterococcus* genus with two isolates identified as *Ec. faecalis* and the other as *Enterococcus* sp. Closely related to *Ec. faecalis*. The spectrum of inhibitory activity of the isolates was evaluated against a range of Gram-positive and Gram-negative bacteria. *Staphylococcus aureus* was the most sensitive indicator tested, whereas *Lactococcus lactis* and *Lactobacillus paracasei* subsp. *paracasei* were the most resistant ones. *Lb. paracasei* subsp. *paracasei* was the unique isolate active against Gram-negative bacteria (*E. coli* and *Salmonella* sp.).

The antimicrobial activity of the bacteriocins produced by the isolates in this work could act as a potential barrier to inhibit the growth of spoilage bacteria (*E. coli* and LAB) and food-borne pathogens (*Listeria* sp., *Staphylococcus aureus*, and *Salmonella* sp.).

**Key words:** lactic acid bacteria, *L. monocytogenes*, bacteriocins.
Screening of polyhydroxyalkanoates-producing bacteria from different environments

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Bioplastics are natural biopolymers that are synthesized and catabolized by various organisms and have certain advantages over petroleum-derived plastic. Bioplastics are lipid in nature and accumulated as storage materials in the form of polyesters of hydroxyalkanoates (PHAs). In this investigation we screened different environments for PHA producing bacteria. Bacterial isolates were obtained by dilution-streaking of nutrient agar (NA) plates. All the bacterial isolates were subjected to an initial Sudan Black B staining to detect the presence of lipid granules in the bacteria. Lipid-positive isolates were then grown in a modified E2 medium to promote accumulation of PHA before the subsequent staining with Nile blue A. The positive isolates were quantified by cryotonic acid assay using UV spectrophotometer. High PHA-producing isolates were selected and were confirmed by GC/MS chromatographic analysis. A total of 132 bacterial strains were screened for isolation of PHA-producing bacteria by Sudan Black B staining. Twenty PHA positive isolates were selected by Nile blue staining from different samples. The selection of the final 20 isolates was based on the highest amount of PHA produced by these isolates in E2 medium with 2% glucose and as measured by U.V. spectrophotometer. Initially, these isolate produced PHA from 0.5 to 1.5 g/l, amounting to about 10-60% of cell dry weight. Among them, the bacterium Bacillus endophyticus BCCS 011 was selected with highest capability for production of PHA (1.5 g/l) and confirmed by GC/MS chromatographic analysis. The GC/MS analysis showed the polymers produced by the selected isolates were polyhydroxybutyrate (PHB).

Solar UV radiation quality and nitrogen starvation induce changes in the commercial carotenoid profile of a Dunaliella bardawil mutant

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Nowadays, it is well-known there is plenty of factors affecting productivity and accumulation of carotenoids in Dunaliella microalgae cultures. Among these factors, optimal photobioreactor design (e.g. open ponds or closed photobioreactors), variations in temperature, nutrients and light conditions are included. Many studies have been focused on the effects that different light intensities incoming microalgal cultures at both pilot or lab scale can produce. However, there is still lack of information about how light quality could modify the carotenoid profile of Dunaliella under natural conditions.

In the present work we show the results obtained after modifying the solar natural UV-radiation spectrum received by batch cultures of a Dunaliella bardawil mutant growing in small closed systems. Cultures were exposed to natural solar radiation conditions in Spain’s southwest coast during May and June. In this period, where productivities are maximal, irradiances are quite high (>1500 μmol.e.m−2.s−1 of ultraviolet radiation) and the temperature does still not affect culture viability negatively. Under such conditions, lutein content per unit of biomass in the absence of UV-radiation (PAR only) was higher (ca. 10%) than that for those cultures which received the entire solar spectrum (PAR plus UV). On the contrary, Dunaliella cultures growing without limitations in light quality (i.e. PAR plus UV) also showed a higher beta-carotene content (ca. 30%); mainly due to the presence of the most energetic UV radiation, i.e., UV-B (280 – 315 nm). Probably, the microalga activates mechanisms to increase the level of this carotenoid in order to dissipate that excess energy.

In addition, another stress factor as nitrogen starvation, normally used to stimulate carotenoids synthesis, was assayed to evaluate possible combined effects on carotenoid production linked to the presence or absence of UV-radiation. In that sense, cultures incubated under nitrogen starvation and UV-radiation showed a specific accumulation of β-carotene up to 15 times higher compared to cultures growing in full culture medium. However, the specific accumulation of lutein in the presence of UV-radiation and nitrogen was 5-fold higher.

Thus, in this context, a possible process strategy to address the specific accumulation of either β-carotene or lutein from the Dunaliella mutant depending on market demands is discussed.

Keywords: Dunaliella; UV-Radiation; lutein; β-carotene; nitrogen starvation; natural conditions

Acknowledgements: This work has been supported by the Ministerio de Educación y Cultura (Proyecto AGL2006-12741) and Junta de Andalucía (Proyecto de Excelencia, AGR-4337).
Synthesis of small RNA-bacteriophage coat protein derived rod-like and spherical mosaic nanoparticles in *Escherichia coli*

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The small RNA bacteriophages are among the simplest known viruses. Their capsid is composed of a single type of coat protein together with one copy of the maturation protein that is required for infectivity. The coat protein alone, when expressed from a plasmid in *E.coli*, assembles into virus-like particles (VLPs) about 25-30 nm in diameter that are morphologically indistinguishable from native virions. The coat protein can be modified by genetically fusing foreign amino acid sequences to it and result in VLPs that display the sequence of interest on their surface. Such nanoparticles can serve as exposition vectors to promote effective antibody response against the introduced sequences, or they can be used to encapsulate different substances and directed to particular types of cells by engineering cell-specific sequences on their surface.

In cases when insertions of foreign sequences in coat proteins cause their inability to assemble into VLPs, the presence of a “helper” wild-type protein can promote assembly into mosaic particles that contain both proteins – natural and fused. In our laboratory, we are primarily working with VLPs derived from coat proteins of RNA phages GA and fr. By using either co-expression of both proteins – natural and fused – or, in the case of C-terminal fusions, translational read-through, we have created a number of mosaic particles, for instance, GA VLPs carrying the N-terminal fragment of stromal cell-derived factor 1 (SDF-1) or fr VLPs carrying a fragment of hepatitis B virus (HBV) preS1 protein on their surface.

The wild-type coat proteins of fr and GA can also co-assemble into mosaic particles of another kind, which are built from two natural coat proteins. Interestingly, in this case in addition to spherical particles, rod-like structures are formed that have the same diameter as the spherical ones but can exceed 1 μm in length. Although this was initially done by in vitro co-assembly of coat proteins, the rod-like structures also form by co-expressing fr and GA coat proteins from a plasmid in *E.coli*. The rods could also be formed from a mixture of wild-type GA coat protein and fr coat protein carrying the HBV preS1 sequence. Therefore besides purely scientific interest, such rod-like nanoparticles have a prospect to be used for engineering new types of nanowires with desired properties.

**Keywords**: VLP; mosaic nanoparticles; rods

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The catalytic potential of bacterial multicomponent monoxygenases ToMO and PH for the synthesis of antioxidants tyrosol and hydroxytyrosol.

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Olive oil is the main fat source in the Mediterranean diet and a growing experimental evidence has been accumulated in the last decades highlighting a connection between the regular incorporation in the diet of this natural products and several health benefits such as a lower incidence of coronary heart disease and of certain cancers. Among natural phenolic compounds found in olive fruits and in virgin olive oil, hydroxytyrosol is certainly one of the most attractive due to several properties such as its antibacterial activity, scavenging of free radicals, protection against oxidative DNA damage and LDL oxidation, prevention of platelet aggregation and inhibition of 5- and 12-lipoxygenases.

As this α-diphenol is not commercially available, much attention has been recently devoted to develop methods aimed at obtaining this compound either from natural sources like vegetative waters or through chemical synthesis. However, both these methodologies show several limitations due to their low yields and the use of toxic reagents.

As a consequence much effort has been dedicated in the last years to the development of bioconversion processes which make use of the metabolic versatility of either purified enzymes or whole microorganisms to perform enzymatic syntheses of industrial interest, with high regioselectivity and stereoselectivity and under mild experimental conditions. In this perspective, the utilization of bacterial multicomponent monoxygenases (BMMs) is of particular interest given the fact that these enzymes catalyze a variety of complex oxidations including monohydroxylation and dihydroxylation reactions of aromatic compounds, which could represent the starting material for the biosynthesis of phenolic antioxidants usually found in olive oil such as tyrosol and hydroxytyrosol.

Among others, the multi-enzymatic systems ToMO (toluene monooxygenase) and PH (phenol hydroxylase) isolated from *Pseudomonas sp.* OX1 are responsible in vivo for the hydroxylation of toxic aromatic molecules which, once activated, are further processed to give molecules that can eventually enter the citric acid cycle. Both ToMO and PH have been extensively studied for the analysis of the molecular determinants responsible for their regioselectivity in the hydroxylation reaction. Recently, a computational model has been developed that quantitatively predicts the effects on regioselectivity of mutations in the active site pocket of the hydroxylase moiety of ToMO, thus allowing the rational design of variants of the enzyme to be used in biosynthesis and biomediation procedures.

In this work, 2-phenylethanol, a cheap and commercially available substrate, has been chosen as the starting material of a biosynthetic pathway which uses ToMO and PH for the catalysis of two steps of hydroxylation to obtain both tyrosol and hydroxytyrosol (see Scheme).

Moreover, an improved computational model was developed, and several variants of ToMO were tested for their ability to convert 2-phenylethanol in tyrosol. ToMO variants E103G-F176V and E103G-F1765 were separately cloned in a PH-containing plasmid to develop an *E.coli* cell-based system endowed with the ability to perform a first and a second hydroxylation reaction on the aromatic ring of 2-phenylethanol to obtain tyrosol and hydroxytyrosol, respectively, as shown in the following scheme.

**Keywords**: monoxygenase; hydroxytyrosol; bioconversion
The potential of thermophilic Fe(III)-reducing prokaryotes to produce novel types of proteinaceous nanowires.

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2 Shewanella species. The studies revealed direct electron transfer from cells to the insoluble electron acceptor to be the key strategy for Fe(III) oxide’s reduction. Moreover, a novel physiological feature among microorganisms has been detected – transfer of electrons via electrically conductive pili called bacterial nanowires. However, the mentioned Geobacter and Shewanella species represent only 2 of ca. 56 genera of Fe(III)-reducing prokaryotes reported so far, and further studies are needed to estimate the ecological role of the detected electron transfer mechanisms and their distribution among different groups of Fe(III)-reducers.

We have investigated the mechanisms for the reduction of amorphous Fe(III) oxide in thermophilic dissimilatory Fe(III)-reducing prokaryotes, which represent ca. 50% of the overall physiological diversity of Fe(III)-reducers known so far. Two organisms of different phylogenetic affiliation have been the subjects of our studies: (1) a thermophilic Gram-positive bacterium Carboxydothermus ferrireducens supporting its growth at 65 °C via dissimilatory reduction of amorphous Fe(III) oxide into magnetite by the strategy of direct cell-to-mineral contact; and (2) a hyperthermophilic archaean Geoglobus acetivorans (optimal growth at 81 °C), recently isolated from the deep- submarine thermal field and depending on insoluble Fe(III) oxide as an electron acceptor. We have detected the formation of pili-like proteinaceous appendages by both of the organisms in response to the presence of insoluble Fe(III) in the culture medium. Electron microscopy and X-ray microanalysis revealed that pili-like structures in C. ferrireducens and G. acetivorans connected the cells with each other and with the insoluble iron oxide to facilitate extracellular electron transfer. Full transformation of Fe(III) oxide into magnetite and the absence of pili production in the cells grown with soluble Fe(III) source indicate the potential electron transfer role of the detected appendages. The presence of the main structural subunits of pili has been genetically evidenced for both organisms. However, no any homology of the pilin-related genes with the previously described ones for Shewanella and Geobacter nanowires have been revealed. Instead, in Geoglobus acetivorans we have detected a highly similar homolog of an archaeal pili domain from Methanococcus maripaludis. The detection of pili in Archaea represents comparatively rare phenomenon and little is known now about their structure and functions.

The obtained data shows that the mechanism of extracellular electron transfer, facilitated by proteinaceous nanowires, could reside to phyllogenetically and physiologically different groups of Fe(III)-reducers. The resulting diversity of electron transferring pili creates an intriguing task of the search for interrelations between structural features of these appendages and their conductive properties. Such investigations could provide insights into poorly understood nature of the conductivity of microbial nanowires, and finally stimulate the development of biotechnologies aimed at production of the nanowires with predefined properties. As an example, the conductivity of the pili produced by thermophilic microorganisms is supposed to exceed the conductivity of the pili, found in mesophiles, due to generally recognized peculiarities in the amino acid composition of thermostable proteins, i.e. increase in the number of charged and aromatic amino acids. Further, the pili of Gram-positive bacteria, in contrast to Gram-negative ones, are characterized by covalent bonds between pilin subunits which could also facilitate electron transfer along the filaments increasing their conductivity. Anyway, much more detailed studies are necessary for understanding of the nature of conduction in microbial nanowires, their exact physiological role and biotechnological potential.

Keywords: thermophiles; microbial Fe(III)-reduction, Gram-positive bacteria, Archaea, nanowires.

Transformation of nerol with Aspergillus niger in Czapek-Dox medium

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Aspergillus niger AHU7120 was cultured in Czapek-Dox medium 3 days, after that, nerol (2mmol) was added to the culture. The culture was continued 21 days from the addition of substrate. α-terpineol and linalool were produced at day 1 and increased until day 7 in the case of α-terpineol and that 3 in the case of linalool, whereas substrate nerol was decreased until day 7 (Fig.1). The formation mechanism of α-terpineol and linalool from nerol or geraniol in the HCl was reported via same deolocalized cation. However, the amount of produced α-terpineol was much larger than that of produced linalool. Therefore, the production mechanism of α-terpineol and linalool was assumed via different intermediates cations. Accordingly, It assumed that α-terpineol was produced via intermediate II and linalool was produced via intermediate III in scheme 1.

Some minor compounds were also produced in this transformation. p-Menthane-1, 8-diol was produced at day 1 and increased until day 8. It can be produced by the addition of H2O to α-terpineol. After 7 days, p-menth 1-8-dien-7-ol was started to produce and increased until day 12. It can be produced by the addition of H2O to α-terpineol. After 7 days, p-menth 1-8-dien-7-ol was started to produce and increased until day 12. It can be produced by the de hydration from α-terpineol followed oxidation of 7-methyl group. 2,6-Dimethyl-3,6-octene-1,6-diol was produced at day 7 and increased until day 9 by the oxidative hydroxylation of linalool. Cis and trans-2,6-Dimethyl-5,7-octene-2-ols were produced at day 8 and increased until day 12 by the hydration and dehydration of linalool.

A few amounts of furan type linalool oxides and pyran type of cyclized compound were produced from linalool. Furan type linalool oxides were produced at day 1 and increased until day 12 by the cyclization of linalool and oxidative hydroxylation of isopropyl group. Pyran type of cyclized compound was produced at day 1 and increased until day 12 by the cyclization of linalool.

Keywords: transformation of nerol, Aspergillus niger, Czapek-Dox medium
Use of volatile acids waste in the production of xanthan gum in a culture of Xanthomonas campestris pv campestris - CBMAI 199 (ATCC 33913)

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The aim of this study was the production of xanthan gum with the use of by-products (VFA) generated in bioprocesses. The world scientific community frantically seeks for new energy sources. Immediately, in parallel, we should concern about the destination of the new waste that will be generated. Second generation wastes (hydrogen and methane) used to be an attractive energy sources, but present with different amounts of volatile acids. The reuse of those acids must be technically and, economically viable: high value of the xanthan gum enables feasible the new energy source: hydrogen. The xanthan gum was produced with short-chain volatile acids (acetate, propionate and butyrate) in substitution of the citric acid present in the medium as suggested by Garcia-Ochoa, 2004. The microorganism used in the studies was the species Xanthomonas campestris pv campestris collection CBMAI – 199 (ATCC 33913). Preliminary studies were done with commercial salts, in batch process carried out in shaker at 28°C and 250 rpm. Three different salts were used to replace the citric acid: (a) 0.0328M sodium acetate, (b) sodium propionate 0.0219M and (c) 0.0164M sodium butyrate. In the first series the best gum production / biomass (g / g) in the corresponding time of culture were Ac (20.6 / 69 h), Prop (31.8 / 47 h) and But (33.8 / 47 h). In concentration of 0.25% the acetate gum showed consistency index of 1.68 and flow index of 0.5. Their consumption of glucose in the growth period of 69 hours was 73%, 39%, 33% and 34%. In another series the test the conditions they were different, including the glucose content lower (26 to 35%) in the assays. The gum production / biomass (g / g) they were lower, corresponding to content lower of substrate. However, the biomass (g / L) increased 30 to 100% in tests.

Keywords Xanthomonas campestris; Xanthan-gum; volatile fatty acids

UV-A mediated modulation of photosynthetic efficiency, xanthophyll cycle and fatty acid production of Nannochloropsis

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Nannochloropsis, a green microalgae, is source for commercially valuable compounds as extensively described and, in particular, is recognized as a good potential source of EPA (20:5ω3), an important polyunsaturated fatty acid for human consumption for prevention of several diseases. Climate change might include variation in the UV levels, as one of the consequences derived from the antropogenic activity. This paper shows the response of Nannochloropsis cultures exposed for 7 days to UV-A added to PAR. Growth rates and photosynthetic activity were assessed to determine the impact of UV-A increased levels on cell growth and basic metabolic activity. Xanthophyll pigments (zeaxanthin and violaxanthin), carotenoids (cantaxanthin and β-carotene) and PUFAs (miristic, palmitic, palmitoleic, araquidonic and eicosapentanoic acids) were measured for assessing the antioxidant response of the microalgae to added UV-A radiation to PAR. The results show that the modulated use of UV-A radiations can lead to increased growth rates which are sustained in time by an increased light transduction activity. The expected antioxidant response to the incident UV-A radiation consisted of increases in zeaxanthin -through an increased activity of xanthophyll cycle- and β-carotene contents (both of them are antioxidant carotenoids) and increases in the SFAs (saturated fatty acids) to PUFAs (polyunsaturated fatty acids) ratio. The results suggest that modulated UV-A radiation can be used as a tool to stimulate value molecules accumulation in microalgae through an enhanced both light transduction process and microalgal antioxidant response, while sustaining cell growth.

Keywords: microalgae, Nannochloropsis, UV-A, xanthophyll cycle, fatty acids

Acknowledgements: This work has been supported by the Ministerio de Educación y Cultura (Proyecto AGL2006-12741).
Xylitol production from dilute-acid hydrolysis of bean group shells

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Xylitol, a five-carbon sugar alcohol, is used as a sweetener in foods and sugar substitute for the treatment of diabetes. Xylitol can be obtained from hemicellulosic fraction of lignocellulosic materials containing D-xylose. Bean group shells, an agricultural waste, is an abundant source of hemicellulose, especially pistachio shells. The hemicellulosic contents of pistachio shells is approx 50% and consists mainly of xylitol. The aims of this study are to optimize the extraction of hemicellulosic sugar xylitol from pistachio shells and to produce xylitol using xylitol derived from pistachio shells.

Xylose production from pistachio (Pistacia vera L.) shells was carried out using 0.1 to 10.0 %(v/v) sulfuric acid. Also, microbial xylitol production from xylose was investigated using Candida tropicalis NBRC 0618. The maximum xylose concentration in the hydrolysate was attained when the pistachio shells were treated at 121 °C for 20 min, using 5.0 % (v/v) sulfuric acid. To remove fermentative inhibitors from the hydrolysate, two different detoxification methods, namely active carbon adsorption and ion exchange resin treatment, were tested. Ion exchange resin treatment using both anion and cation exchange resin were found to be effective for detoxification of the hydrolysate. Maximum xylitol productivity was 0.086 g/L/h which is 4.8 higher than the value obtained from untreated samples.

Keywords: pistachio shells; xylitol; hydrolysis

Potential of microbial consortium for biological treatment of the effluent from cassava flour production

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Wastewaters from the production of cassava flour contain high organic material and hydrogen cyanide. These pollutants need to be degraded before being launched in the water resources. The aim of this work was to obtain a microbial consortium from the effluent of cassava flour production and to investigate its potential for the biological treatment of this effluent.

Experiments were carried out in a bioreactor with the effluent from cassava flour production in the presence of ammonium sulfate 0.8 %, at 28 – 30 °C, 1vvm and 200 rpm. Three pulses of 10% (v/v) of the effluent, supplemented with the nitrogen source, were weekly added to the bioreactor after seven days of cultivation. The microbial growth was determined by aerobic plate count of bacteria, yeasts and filamentous fungi. Enzyme activities and chemical oxygen demand were determined.

The concentration of carbohydrates (2,6%), proteins (1,8%) and lipids (0,4%) in the effluent stimulated the growth of the autochthon microorganisms, under experimental conditions. The maximum growth of bacteria in the consortium reached 10⁸ CFU/mL at the stationary phase. Maximum activities of amylases, cellulases, lipases and proteases was detected after seven days of cultivation. The organic matter was aerobically metabolized by the autochthon microorganisms of the consortium, during 48 h, at 30 °C and 150 rpm. Reductions of the chemical oxygen demand (80%) and hydrogen cyanide (28%) were determined after the biological treatment of the effluent.

The microbial consortium obtained from the effluent has potential for the biodegradation of the effluent samples in aerobic condition.

Keywords: microbial consortium, cassava flour production, biodegradation.
Bioprospection of microorganisms for lipase production using an industrial waste as carbon source

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Scientific research has been developed for the treatment of industrial effluents and the reuse of its nutrients in the cultivation of microorganisms to produce metabolite of economic value. "Manipueira" is the principal component of the effluent from the cassava flour production. The high organic material in the effluent causes negative impact in the environment. The aim of this study was the production of lipases - one of the most important biocatalysts for biotechnological applications – by samples of microorganisms isolated from the effluent of the cassava flour production.

Samples of bacteria, yeasts and filamentous fungi were isolated from this effluent. The microorganisms were inoculated in 500 mL Erlenmeyers flasks, using 300 mL working volume of the medium: 2 % "manipueira", 1 % olive oil and 0.25 % ammonium sulfate. The experiments were carried out in a shaker at 150 rpm, at 28 °C, during 8 days. The samples were centrifuged and the supernatants were used to determine the pH (potentiometry) and the lipolytic activity. The enzyme activity was determined with paranitrophenilpalmitate (pNPP), dissolved in isopropanol in the presence of Triton-X100 and gum arabic in buffer Tris-HCl 50 mM pH 7.0.

The lipase production increased with the cultivation time for 45 % of the microorganisms investigated. Some microbial samples showed different peaks of lipolytic activities. A filamentous fungus sample showed atypical behavior of lipase production: on the second day of the cultivation, the maximum activity was 7.9 IU/L and this value decreased gradually over the time. The maximum lipase production reached 10.1 IU/L at pH 5.6 after 8 days by a bacteria sample while the maximum productivity was 3.95 IU/L.d obtained at pH 3.9 by a filamentous fungus sample.

More research needs to be stimulated on bioprospection of microorganisms for enzymes production with the reuse of industrial waste, avoiding environmental impact and reducing costs for biotechnological processes.

Keywords: bioprospection, microorganisms, industrial waste, lipase production.

Effectiveness of N-acetyl cysteine (NAC) on prevention and eradication of S. epidermidis biofilms.

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Background: The infections related with artificial devices implanted in the human body suppose a serious problem at the present time. These infections are very frequent and they are almost always produced by Staphylococcus spp. Previously, the efficacy of N-acetilcystein (NAC) to decrease bacterial PIA/PNAG-dependent biofilm, has been demonstrated. So, in this work we will confirm these data and also studied the degradation extracellular polysaccharides (EPS) in mature biofilm.

Methods: All total of 6 biofilm producing S. epidermidis strains were used to investigate the activity of N-acetylcysteine (NAC) against biofilms developed. The quantitative measurement of biofilm production and the Slime Index (SI) in presence of NAC subinhibitory concentrations was determined. The effect of NAC on different biofilm components of S. epidermidis by microtter detachment assay was carried out. The PIA/PNAG production by Dot-blot and hemagglutination inhibition was analyzed. The bacteria included in biofilm after treatment with NAC would be quantified using BacTiter-Glo™ Microbial Cell Viability Assay and mature biofilms were observed through fluorescent microscopy.

Results: In all strains we confirmed the reduction of PIA/PNAG-dependent biofilm in the presence of N-acetylcysteine (NAC) against biofilms developed. The quantitative measurement of biofilm production and the Slime Index (SI) in presence of NAC subinhibitory concentrations was determined. The effect of NAC on different biofilm components of S. epidermidis by microtter detachment assay was carried out. The PIA/PNAG production by Dot-blot and hemagglutination inhibition was analyzed. The bacteria included in biofilm after treatment with NAC would be quantified using BacTiter-Glo™ Microbial Cell Viability Assay and mature biofilms were observed through fluorescent microscopy.

Conclusions: Our result suggesting that decrease of biofilm development in S. epidermidis strains is due to the inhibition of PIA/PNAG synthesis and accumulation on the bacterial surface. For this reason, NAC is interesting candidate for use as inhibitor of formation of bacterial biofilms on medical device.

Keywords: Staphylococcus epidermidis; Biofilm; Medical-device infections; N-acetyl cysteine.
**An in vivo study on the fungicidal effects of PAA (Per acetic Acid) against phytopathogenis fungi**

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The peroxide of acetic acid, is one of the most important organic peroxides with wide spectrum of antimicrobial activity. Mixed peracid or peracetic systems are made with peracetic acid (PAA), hydrogen peroxide and acetic acid. There is a growing to develop alternative chemicals recognizing as safe, less harmful to human health and the environment, for controlling phytopathogenic fungi. Peracetic acid (PAA) has potential as a disinfectant of low environmental impact for glasshouse hydroponic systems and other horticultural applications.

The objective of this study was evaluate peroxyacetic acid (PAA) as alternative to Metam sodium in the control of soilborne diseases in hydroponic substrates. Four pathosystems were studied: *Pythium aphanidermatum*-cucumber, *Phytophthora parasitica*-tomato, *Fusarium oxysporum f.sp. radicis-cucumerinum*-cucumber and *Sclerotinia sclerotiorum*-zucchini. Also fungicide effect of PAA was compared with metam sodium at common dose rates used in substrates disinfection.

The results demonstrated that PAA could be a good alternative disinfection method for elimination of fungi in substrates. The most fungicidal activity was observed against the *Fusarium oxysporum f.sp. radicis-cucumerinum*. The different observed in antifungal activity of PAA may suggest susceptibility of various phytopathogenic fungi against oxidation potential of PAA. Limited data are available on the control of phytopathogenic fungi in field conditions by PAA, so more studies are needed to test other important phytopathogenic fungi and further study are also needed to test it phytotoxicity towards host plants.

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**Catalytic Promiscuity of Thermostable T1 Lipase from Geobacillus zalihaii by Metal Substitution**

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The research of metalloenzyme has become an active area in biochemistry and biophysics. For many experimental approaches, large quantities as well as high concentration of protein are needed. Purification for improving fusion lipase without signal peptide has been accomplished using affinity chromatography glutathione-sepharose. The crude lipase was obtained from cell lysate of recombinant clone *E. coli* BL21 (DE3)pLysS (pGEX/T1S). The purification yields of 42.66 and 44.37% were obtained for T1 fusion lipase (GST + T1 mature lipase) without signal peptide and T1 mature lipase (T1 lipase without signal peptide), respectively. In the present work, we explore the adjacent amino acids to Zn2+. There are three Histidine groups bind to Zn2+. Histidine is an essential amino acid that has as a positively charged imidazole functional group which is a common coordinating ligand in metalloproteins and is a part of catalytic sites in certain enzymes. Metal removal experiment of 5.72mg of protein was done using 2,6-pyridinedicarboxylate as the killer agent. The specific activity of pure T1 lipase increased three folds to 30.66 U/mg after serial dialysis to remove the zinc from its structure. This could be due to the changes in its structure that makes T1 become more active when the zinc has been removed. Later, metal ion substitution of Zn2+ with transition earth metals was performed and evaluated in term of its biophysical analysis, thermostability, structural-function relationship, enantioselective and protein crystallization studies.

**Keywords**: metalloenzyme, T1 lipase, enzymes, biocatalysis, metal binding
Design of surfaces response analysis of $k_{La}$ depending of aeration and agitation in 14 L bioreactor

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Oxygen is the most essential requirement for aerobic bioprocesses. The microbial growth in a bioreactor depends upon the oxygen transfer rate (OTR). The OTR is widely used to study the growth behavior of microbial and plant cell cultures. The mass transfer coefficient ($k_{La}$) determines the magnitude of the OTR. There are many techniques for measuring oxygen concentration and OTR in bioreactors. In the present work we showed the experimental design to use the surface response analysis $k_{La}$ as a function of aeration and agitation in 14 L bioreactor. The goal of surface response design, consist in optimize the experimental condition to generate a model for a region denominate surface, and this surface was predicted from controlled factors. Based on the limits of the factors used (100 a 600 rpm y 0.5 a 1.5 vvm volumen air/volumen liquid. min) we use “Face Centered” design (Figure 1). The surface design response was run in two replicates in the center and 8 experiments as describes in (Figure 2). The software used was MINITAB® Release 14.12.0.

The experiment was run in a 14 L bioreactor (New Brunswick), fill to 10 L, and operated according to experimental design. Between each run the oxygen was eliminated of the reactor with nitrogen flow, to the concentration of oxygen was zero. After this the aeration was started at indicated rpm. Using a Clark electrode, oxygen concentration was reading in time. With this data factor $k_{La}$ was calculated for each condition (Figure 3). The software analysis of $k_{La}$ data for 10 experiments, we obtained the general equation and a graphic of the surface response (Figure 4), which shown a correlation factor of 99%. With this result is possible estimate the $k_{La}$ of the reactor with high precision with minimum number of experiment.

Ecodynamics and impact of copper on microbial communities in a vineyard soil variably amended with organic matter

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In a coupled microcosm and lysimeter study, the effect of the soil organic status (SOS) on copper outcome and impact was investigated in a vineyard soil that had been amended with varying types of organic matter during a previous long-term field experiment. Soil microcosms and lysimeters were contaminated at 240 mg Cu kg⁻¹ and incubated for 6 months. Copper distribution and dynamics were assessed in the solid matrix by size fractionation and sequential extraction procedures and in the soil solution by measuring total and free exchangeable copper concentrations. Copper bioavailability was also measured with a whole-cell biosensor. Variations in microbial communities were assessed by means of biomass-C measurements and characterization of genetic structure using ARISA (Automated-Ribosomal-Intergenic-Space-Analysis). Results showed that copper distribution, speciation and bioavailability are strongly different between organically amended and non-amended soils and largely driven by the SOS. Unexpectedly, in solution, bioavailable copper correlated with total-copper but not with free-copper, suggesting that non-free copper remains bioavailable to microorganisms. Similarly the observed differential copper impact on microorganisms suggested that organic matter controlled copper toxicity through the control of both copper speciation and the structure of the microbial communities in the different soils. Bacterial-ARISA modifications corresponding to the soil enrichment in Actinobacteria, also correlated with the estimated metal bioavailability. Contrarily, biomass-C and Fungal-ARISA measurements did not relate trivially to copper speciation and bioavailability suggesting that the composition of the initial indigenous-soil communities, which is amendment-specific, controls its sensitivity to Cu.
Evaluación de antagonismo en la fíllosfera de tomate

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In vitro assessed antagonistic 5 of isolated group's capacity bacterial and fungal 68 of the healthy tomato plants from organic production greenhouses leaves phyllosphere. The isolated were faced pathogens Botrytis cinerea, Fusarium oxysporum fsp lycopersici (FOL), Fusarium oxysporum fsp radicis lycopersici (FORL), Mycosphaerella pinodes, Phytophthora parasitica, Pythium aphanidermatum y Verticillium dahliae. The results show that 5 of the bacterial isolates showed antagonistic activity to pathogens P. aphanidermatum and FORL and fungal 18 to B. cinerea, P. aphanidermatum, M. pinodes and V. dahliae.

Keywords: antagonism, endophytes, phyllosphere, in vitro.

Experimental investigation and modelling of biofilm growth and hydrodynamic/biomass interaction in a granular bioreactor applied to phenol removal

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The study of biofilms in porous media spans a range of environmental applications going from subsurface ecosystems to industrial bioreactors. The development of bacterial biofilms in porous media is driven by complex processes that involve fluid flow, nutrient transport, microbial ecology and/or biotransformation. In practice, in order to describe the biological behaviour of a bio-reactor that usually ends up with porous media clogging, it is almost impossible to account for all these biophysical processes at the pore scale, and continuum macroscopic modelling approaches are usually preferred although not satisfactorily from a pure mechanistic point of view.

This paper aims at deriving macroscopically equivalent medium from the description of biophysical mechanisms occurring at the microscopic scale using an homogenization method of multiple scale expansions for periodic structure. For this purpose, a medium scale pilot reactor has been developed, which, a biofilm of Pseudomonas putida was grown under varying controlled conditions. Bacterial growth was indirectly monitored through macroscopic pressure drop measurements until complete bio-clogging of the porous medium. Our modelling approach was then applied to simulate observed data and in particular to indirectly derive the evolution of the macroscopic permeability through pressure drop profiles. The modelling results were then validated by measured profiles of bacterial biomass and pollutant and oxygen concentration. The results were compared with outputs obtained with simple and classical 1D models and discussed in the light of the main processes occurring at the microscopic scale.
First Report of Vegetative Compatibility Group 0136 of *Fusarium oxysporum* f.sp. *melonis* in Colima State, Mexico

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*Fusarium oxysporum* f.sp. *melonis*, the causal agent of the Fusarium wilt of melon (*Cucumis melo* L.), was described in Colima State’s melon crops during years 2001, 2002 and 2003 by de Cara et al. (2004). They studied 4 soil samples obtaining 31 isolates of *F.o.* f.sp. *melonis*. This 31 isolates were inoculated on melon plants to know their pathogenicity. The results of this inoculation tests allowed to de Cara et al. to affirm that all isolates were race 1 of *F.o.* f.sp. *melonis*. Because of the homogeneity we wondered about isolates’ genetic variability. Then, following the same methodology employed to determinate VCGs of *F.o.* f.sp. *radicis-cucumerinum* (García-Alcázar et al., 2006), we studied isolates of *F.o.* f.sp. *melonis* from Colima State (Mexico). To begin the study 20 isolates were selected and 4 monosporic isolates were obtained from each isolates. Then 2 monosporic isolates was selected in order to obtain nitrate nonutilizing mutants (*nit* mutants). *nit* mutants were obtained from 80% of the isolates. After phenotypic identification of *nit* mutants, they were paired themselves and with the testers isolates in order to know their self compatibility and compatibility groups. Two isolates (their two monosporic isolates) were determined as HSI (Heterokaryon Self Incompatibility). The rest of isolates, 15, were determined as HSC (Heterokaryon Self Compatibility). All of them were included in VCG 0136. This VCG has already been cited in Mexico by Jacobson and Gordon (1991). It means all isolates are natives, so they haven’t been introduced from foreign countries. Moreover, as all isolates belong to the same VCG, they are able to anastomose cells and form heterokaryons, so there is possibility of genetic recombination on this somatic cells (Puhalla y Spieth, 1983).

Hypocholesterolemic Effects of *Lactobacillus* Strain Isolated from Blondo (Waste of virgin coconut oil) Observed in Broiler Cholesterol Contained Diets

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Order to evaluate the hypocholesterolemic effect of *Lactobacillus* an isolate from blondo in the broiler were fed cholesterol containing diets supplemented with fermented cultured with TMC 0409. No significant inhibitory effect was observed in the broiler fed after 14 days feeding. But the increase of serum cholesterol was suppressed (36%) in the broiler fed TMC 0409 lyophilized cells. Total cholesterol, HDL-cholesterol, triglyceride and phospholipid in serum, neutral steroid and bile acids in feces, cholesterol and triglyceride in liver were analyzed in the rats fed TMC 0409 cells after 14 days feeding. Total cholesterol and phospholipid were significantly decreased compared to those of control group (*P*<0.01), however HDL-cholesterol was significantly increased (*P*<0.05). Serum triglyceride was also decreased but the difference was not significant. From these results, the inhibition of increase of serum cholesterol in broiler fed TMC 0409 cells was considered to have resulted from the reduction of LDL+VLDL-cholesterol. Meanwhile, the accumulation of triglyceride in the liver was inhibited significantly compared with the control group (*P*<0.05), and the excretion of bile acid in the feces was also enhanced significantly (*P*<0.01). These results imply that strain TMC 0409 can alter the serum cholesterol concentration, especially LDL +VLDL-cholesterol by enhancing the excretion of bile acids in the faces.

Keywords: hypocholesterolemic, *Lactobacillus*, broiler
In situ identification of bacteria involved in Polycyclic Aromatic Hydrocarbon biodegradation and proteomic analysis of associated enzymes

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Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous and persistent contaminants in the environment, which are of concern because of their mutagenic and/or carcinogenic effects. Among the treatments proposed to remove pollution from contaminated sites, bioremediation, which uses microorganisms to degrade organic contaminants, appears attractive because it is cost-effective and it preserves ecosystems. However, bioprocesses currently implemented to remove stable and very hydrophobic pollutants such as PAHs, are slow and incomplete. Understanding biodegradation processes in situ is therefore crucial to improve bioremediation.

Previous studies on PAH-degrading bacteria isolated from polluted sites, essentially Mycobacterium and Sphingomonas strains, contributed to a better understanding of the metabolic pathways and the enzymes involved in PAH degradation [1-3]. Ring-hydroxylating dioxygenases, which catalyse the initial ring attack on PAH, have been characterised [2]. However, culture-dependent approaches do not take into account factors that affect the fate of PAHs in soil, such as competition among microorganisms or the bioavailability of pollutants. Moreover, since less than 10% of soil bacterial species are cultivable, bacterial strains studied in the laboratory may not be major protagonists of in situ pollutant degradation.

In recent years, approaches based on stable isotope probing (SIP) have been widely applied to identify bacteria responsible for the degradation of organic compounds in soils or sediments. Most SIP methods use 13C-labelled substrates to target active microorganisms. Bacteria able to metabolize labeled substrates incorporate 13C and can be identified through the analysis of biomarkers (16S rRNA or specific catabolic genes) after isolation of 13C-DNA [4].

In this study, we have undertaken a SIP analysis of PAH-degrading bacteria from a constructed wetland collecting road runoffs, using 13C-phenanthrene as a probe. 13C-DNA recovered from soil is used to amplify and analyze by sequencing 16S rRNA and dioxygenase genes. In addition, Single Strand Conformation Polymorphism [5] of a variable region of 16S rRNA is used to highlight changes in the whole bacterial community during incubation with PAHs. Preliminary data obtained using these DNA-based molecular approaches will be presented.

Moreover, we are testing the possibility of identifying 13C-labelled proteins recovered from soil by metaproteomic analysis. The approach involves extraction of bacteria from soil, separation of protein homogenates by SDS-PAGE, and shotgun proteomic analysis through LC-MS/MS. The challenge is to identify key enzymes involved in PAH degradation directly from environmental samples.

The molecular methods developed in this study may eventually serve as diagnostic tools for monitoring mitigation of PAH pollution in soils upon treatments by bioremediation.

References

Keywords: stable isotope probing, PAH degradation, metaproteomics, single-strand conformation polymorphism, mass spectrometry
Molecular Characterization of Bacteria Acetic Lactat Isolated from Blondo (Waste Virgin Coconut Oil)

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Specific multiplex PCR assay based on the amplification of parts of the 16S rRNA gene was designed. Primers derived from variable regions of the 16S rRNA provided a means of easily differentiating the species Lactobacillus. They could be clearly discriminated from the phylogenetically related species known to be present in Blondo. Other strains isolated together with Lactococcus plantarum an industrial sourdough fermentation could be clearly separated from these species by comparative sequence analysis and construction of a specific PCR primer. For a fast identification a DNA isolation protocol based on the ultrasonic lysis of cells from single colonies was developed. To demonstrate the potential of such techniques for tracking these organisms in a laboratory-scale fermentation, we combined the specific PCR assay with direct DNA extraction from the organisms in the blondo without previous cultivation.

Key words: BAL, blondo, PCR

Optimization of Lipase-Catalyzed Production of Succinic Acid Ester Using Central Composite Design Analysis

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Esters of succinic acid are extensively used as flavor enhancers in food products and as stabilizers in pharmaceuticals. They are also used as ingredients in solvents, paints, deters, plastics, fuel additives and fabrics. The future for succinates lies in utilizing them in creating innovative biopolymers like polybutylene succinate. Succinates are traditionally manufactured from petrochemicals through expensive processes using a chemical catalyst. The chemical method involves some problems such as high reaction temperature, toxic and corrosive catalysts, complex and expensive reaction setup, large amounts of raw materials due to the un-selectiveness of the process and high waste generation. The use of enzymes as “green” alternatives to produce these high value added esters may offer significant superiorities because of milder reaction conditions, higher selectivity and specificity, lower energy requirement and much purer products. In this work, modeling and optimization of enzyme-catalyzed production of dioleyl succinate was performed using Novozyme 435. Response surface methodology (RSM) and central composite design (CCD) were employed to evaluate the effects of several reaction parameters including reaction time, temperature, enzyme amount and molar ratio of oleyl alcohol to succinic acid on percentage conversion of dioleyl succinate ester. Temperature was the most important variable of the four factors. 99% of ester conversion was predicted at the optimum conditions of enzyme amount 77 g/L, alcohol/acid molar ratio 4.5:1, temperature 55°C, and reaction time 72 min. The model generated is able to predict the percentage of production yield in any given conditions. The obtained optimum conditions can be used to scale up the process in bioreactor.

Keywords: lipase, biocatalysis, succinic, ester, response surface methodology
Studies on siderophore production of Erwinia aphidicola and E. persicina.

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The purpose of this research was to study the relationship between the production of siderophores by E. persicina and E. aphidicola and the pathogenicity caused in Phaseolus vulgaris. Two tests were carried out, the first centred on studying in vitro siderophores production by different isolates of E. persicina and E. aphidicola from beans greenhouse producers through the colorimetric method CAS. Subsequently, different isolates of E. persicina and E. aphidicola producers of siderophores were inoculated in bean plants to determine their involvement in pathogenesis, watching, if alone, they caused any symptoms using the Detached leaf technique. The results of the test in vitro showed that Erwinia persicina isolates from bean and tomato plants (ATCC 49742 and DSMZ 19328, respectively) showed great differences in the production of siderophores. These bacterial isolates were used as a control, because at the beginning of the test these isolates from bean plants with symptoms of chlorosis and necrosis were identified as E. persicina and not as E. aphidicola. In addition, all the isolates of Erwinia aphidicola and the isolate from the aphid Acyrthosiphon pisum (DMZ 19347T) were producers of siderophores. The production of siderophores could not be correlated with the symptoms in the leaves by the technique “Detached leaf” because Pantoea agglomerans did not produce siderophores and showed similar symptoms on the leaves.

Transmission of Erwinia aphidicola on bean (Phaseolus vulgaris) seeds

Santos M., Marín F., Díaz F., Carretero F., Tello J.C.

Since late 2003, has developed in bean crops under plastic in southeastern Spanish, bacteriosis causing a shrinkage in production by over 50%. Santos et al. (2009) identified as the causative agent of the disease the bacterium Erwinia aphidicola, which until then had never before been described as pathogens.

The aim of this work is to determine the possible transmission of bacteriosis through seed. It has 120 seed from commercial bean, cv. “Donna”, the same batch as that used in one of the affected farms and 120 seeds from the fruit of diseased plants of the same farm.

Samples tegument and cotyledons of the seed and leaves of plants originated from them, were analyzed by DAS-ELISA technique. Have also been isolated all bacteria present in the cotyledons of the seed trade. These isolates were analyzed by serological, biochemical and molecular tests.

The PCR amplification and subsequent sequencing of a fragment of 660 bp of dnaJ area of the genome of the bacteria, confirmed the presence of the phytopathogen Erwinia aphidicola in two of the analyzed seeds, which is sufficient for the disease originated in the suitable environmental conditions.

Keywords: Erwinia aphidicola, Phaseolus vulgaris, transmission, seeds