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1 **Pectin degrading enzymes in yeasts involved in fermentation of *Coffea arabica* in**
2 **East Africa**

3
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1 **Abstract**

2 The ability of six strains of *Pichia anomala*, four strains of *Pichia kluyveri* and two
3 strains of *Hanseniaspora uvarum* predominant during coffee processing to produce
4 polygalacturonase (PG), pectin esterase (PE) and pectin lyase (PL) in yeast
5 polygalacturonic acid medium (YPA) and in coffee broth (CB) was studied. For
6 comparison, a reference strain of *Kluyveromyces marxianus* CCT 3172 isolated from
7 cocoa and reported to produce high amount of PG was included.

8 Initial screening of PG activity using YPA medium showed that *K. marxianus* CCT
9 3172, *P. anomala* S16 and *P. kluyveri* S13Y4 had the strongest activity. Enzymatic
10 assays showed that the four yeast species secreted PG, but none of the yeasts
11 investigated was found to produce PE or PL. *P. anomala* S16 and *P. kluyveri* S13Y4
12 were found to produce higher amounts of PG when grown in CB than in YPA. When
13 *K. marxianus* CCT 3172, *P. anomala* S16 and *P. kluyveri* S13Y4 were grown in YPA
14 broth adjusted to pH of 3.0-8.0 and incubated at temperatures of 15-40 °C, the three
15 yeast species secreted the highest amount of PG at pH 6.0 and at 30 °C. For PG secreted
16 by *K. marxianus* CCT 3172 and *P. anomala* S16, the optimum pH and temperature for
17 the enzymatic activity were 5.5 and 40 °C, respectively. On the other hand, PG
18 produced by *P. kluyveri* S13Y4 showed the highest activity at pH 5.0 and 50 °C.

19 Significant differences in the extracellular activity of PG were found between the yeasts
20 species as well as between strains within same species. High amounts of PG were
21 produced by two strains of *P. anomala* and *P. kluyveri*. It is therefore likely that strains
22 of those two species may be involved in the degradation of pectin during coffee
23 fermentation.

- 1 Key words: Coffee, *Pichia anomala*, *Pichia kluyveri*, *Hanseniaspora uvarum*,
- 2 polygalacturonase.

1 **1. Introduction**

2 Coffee is mainly grown in tropical and subtropical regions but is consumed worldwide.

3 A large number of species of the genus *Coffea* have been identified. The commercial

4 coffee beans belong to the two species *Coffea arabica* and *Coffea canephora* var.

5 *robusta*. To separate beans from pulp coffee is processed by dry or wet method. The dry

6 method is mainly used for *robusta* coffee, which has a thin pulp that allows direct

7 drying (Fowler et al., 1998). In general, cherries of *Coffea arabica* worldwide are

8 processed by the wet method. However, more than 80 % of arabica coffee in Brazil,

9 Yemen and Ethiopia are processed by the dry method (Brando, 2004). In wet processing

10 of coffee, the ripe coffee cherries are pulped followed by fermentation and drying

11 (Fowler et al., 1998).

12 It is a main aim of coffee fermentation to remove the pectineous mucilage adhering to

13 coffee beans. According to studies that have been carried out, disagreements about

14 which microorganisms is responsible for pectin degradation exist. Vaughn et al. (1958)

15 examined dry and semi-dry processed Brazilian coffee and found mainly Gram-negative

16 bacteria with pectinolytic activity belonging to the genera *Aerobacter* and *Escherichia*.

17 In addition, pectinolytic species of *Bacillus* and a variety of pectinolytic filamentous

18 fungi were isolated. From wet processed coffee in Hawaii, species of *Erwinia*,

19 *Escherichia* and most commonly *Erwinia dissolvens*, were isolated to which

20 degradation of mucilage was related (Frank et al., 1965). On the other hand,

21 *Kluyveromyces marxianus*, *Saccharomyces bayanus*, *Saccharomyces cerevisiae* var.

22 *ellipsoideus*, and *Schizosaccharomyces* species, which were found to have pectinolytic

23 activity, were isolated from fermented robusta coffee in India (Agate and Bhat, 1966).

1 Pectin is a complex heteropolysaccharide composed of D-galacturonic acid residues
2 joined by α -1.4-linkages, which form homogalacturonan chains (Be Miller, 1986).
3 Enzymes that act on pectin molecules include the pectinesterases (PE) and the
4 depolymerases. PE are able to de-esterify pectin by hydrolysis of the methyl ester
5 group, while depolymerases split the main chain (Be Miller, 1986). The depolymerases
6 are divided into polygalacturonases (PG), which cleave the glycosidic bonds by
7 hydrolysis, and lyases (PL), which break the glycosidic bonds by β -elimination at
8 esterified D-galacturonic acid units. Pectinolytic enzymes from yeasts are mainly endo-
9 PG; they have been reported in *Rhodotorula* spp. (Vaughn et al., 1969), *Cryptococcus*
10 *albidus* (Federici, 1985), *K. marxianus* (Lim et al., 1980; Barnby et al., 1990; Schwan
11 and Rose, 1994; Schwan et al., 1997), *S. cerevisiae* (Blanco et al., 1994), and several
12 species of *Candida* (Call et al., 1985; Sanchez et al., 1984; Stratilova et al., 1998).
13 The aim of the present work was to study the ability of *P. anomala*, *P. kluyveri* and *H.*
14 *uvarum* predominant during wet processing of arabica coffee in Tanzania (Masoud et
15 al., 2004) to produce pectinolytic enzymes in a laboratory substrate as well as in coffee
16 broth. Furthermore, the effects of pH and incubation temperature on the production of
17 polygalacturonase and its activity were also investigated.

18 **2. Materials and methods**

19 **2.1 Cultures**

20 Yeasts used in this study were obtained from arabica coffee samples collected from the
21 Arusha region, Tanzania (Masoud et al., 2004). They included six strains of *P. anomala*
22 (S12, S13, S14, S15, S16, S17), four strains of *P. kluyveri* (S4Y3, S7Y1, S8Y4, S13Y4)
23 and two strains of *H. uvarum* (S3Y8, S15Y2). One strain of *K. marxianus* CCT 3172,

1 which was isolated from cocoa and has been reported to have a strong pectinolytic
2 activity (Schwan and Rose, 1994) was also included.

3 **2.2 Screening for polygalacturonase activity**

4 The yeasts were propagated in 25 ml of malt yeast glucose peptone (MYGP) broth [3.0
5 g yeast extract (Difco, Detroit, MI, USA), 3.0 g malt extract (Difco), 5.0 g bactopectone
6 (Difco), 10.0 g glucose (Merck, Darmstadt, Germany) per 1 liter distilled water] at 25
7 °C for 48 h. After propagation cells were harvested by centrifugation at 3000 x g for 10
8 min, and resuspended in diluent saline peptone (SPO) [0.1 % (w/v) bactopectone
9 (Difco), 0.85 % (w/v) NaCl (Merck), 0.03 % (w/v) Na₂H₂PO₄, 2H₂O (Merck), adjusted
10 with 1.0 M NaOH (Merck) and 1.0 M HCl (Merck) to pH 5.6]. Cell concentrations were
11 estimated by microscopy using a counting chamber (Neubauer) and the suspensions
12 were diluted to final concentrations of 10⁶ cells/ml. Screening for pectinolytic activity
13 was modified from a procedure described by Zink and Chatterjee (1985). Three spots of
14 10 µl of each yeast suspension were placed on plates of polygalacturonic acid specific
15 medium [7.0 g yeast nitrogen base (YNB) (Difco), 5.0 g glucose (Merck), 5.0 g
16 polygalacturonic acid (Sigma), 20 g agar (Difco) per 1 liter distilled water]. The plates
17 were incubated for 48 h at 25 °C and then flooded with 6.0 M HCl (Merck), where clear
18 halo around yeast colonies indicated pectinolytic activity. The experiment was done in
19 triplicates.

20 **2.3 Polygalacturonase enzyme assay in yeast polygalacturonic acid broth (YPA)** 21 **and in coffee broth (CB)**

22 Yeasts were grown in 50 ml of yeast polygalacturonic acid medium (YPA) [7.0 g YNB
23 (Difco), 5.0 g glucose (Merck), 5.0 g polygalacturonic acid (Sigma) per 1 l distilled
24 water adjusted to pH 5.5 with 1.0 M NaOH (Merck)]. Yeasts were also propagated in 50

1 ml of coffee broth (CB) [20 g grounded green coffee beans (Levi Farm, Arusha,
2 Tanzania), 5.0 g glucose per 1 l distilled water adjusted to pH 5.5 with 1.0 M NaOH
3 (Merck)]. Yeast cultures were incubated on a rotary shaker at 30 °C with for 48 h. Then
4 yeast cells were centrifuged at 7000 x g for 20 min at 4 °C. The cell free supernatant
5 was used for PG and the other enzymatic assays described below. For determination of
6 PG activity, a reaction mixture composed of 0.5 ml of supernatant, 0.5 ml of 0.5 %
7 (w/v) polygalacturonic acid in 0.05 M sodium acetate buffer (pH 5.5) and 9.0 ml of 0.05
8 M sodium acetate buffer (pH 5.5) was prepared and incubated in a water bath at 45 °C
9 for 1 h. PG activity was determined by estimation of the amounts of reducing sugar
10 groups as described by Miller (1959). 1.0 ml of the reaction mixture was added to 1.0
11 ml of 3, 5-Dinitrosalicylic acid (DSN) reagent [1 % (w/v) DSN (Merck), 0.2 % (w/v)
12 phenol (Merck), 0.05 % (w/v) sodium sulfite (Merck) and 1 % (w/v) sodium hydroxide
13 (Merck)] and boiled for 15 min then cooled under tap water. To stabilize colour, 1.0
14 ml solution of 40 % (w/v) potassium sodium tartarate (Merck) was added subsequent to
15 colour development and before cooling. After cooling the optical density of resulting
16 coloured mixture was measured spectrophotometry at 575 nm. Galacturonic acid
17 (Sigma) was used as a standard. One unit of PG activity was defined as the amount of
18 the enzyme which catalysed the formation of 1.0 µmol of galacturonic acid per min at
19 45 °C. The enzyme activity was expressed as units per milligram dry weight per ml
20 (U/mg DW/ml) of the supernatant. The experiment was done in duplicates for each
21 yeast strain.

22 PG was also investigated when yeasts were grown in YPA without addition of glucose
23 or in YNB with glucose but without addition of polygalacturonic acid.

1 **2.3.1 The effects of pH and incubation temperature on production of**
2 **polygalacturonase by yeasts**

3 Yeasts were propagated in YPA medium at pH values of 3.0-8.0 and incubated at
4 temperatures of 15-40 °C for 48 h. Production of PG by yeasts was determined as
5 described above.

6 **2.3.2 The effects of pH and temperature on polygalacturonase activity**

7 PG activity was determined at different pH values by incubation of the reaction mixture
8 described above with different phosphate buffers pH 3.0-8.0. Furthermore, the effect of
9 incubation temperature of the reaction mixture on PG activity was investigated at 10-70
10 °C.

11 **2.4 Assay for pectin esterase activity**

12 A solution of 1 % (w/v) of pectin (47 % esterified) (Sigma) in 0.1 M sodium chloride
13 (Merck) was adjusted to pH 7.5 with 0.5 M NaOH (Merck). Quantities of 0.5-5.0 ml of
14 yeasts free supernatant prepared as described above were added to 20 ml of the pectin
15 solution and the pH was maintained 7.5 for 30 min by addition of 0.02 M NaOH
16 (Merck). The enzyme activity is proportional to the volume of NaOH added (Barnby et
17 al., 1990). The experiment was done in duplicates for each yeast strain.

18 **2.5 Assay for pectin lyase activity**

19 One half ml of the yeast free supernatant prepared as described above was added to 0.25
20 % (w/v) pectin in 0.1 M Tris-HCl buffer, pH 7.5. The activity was determined
21 spectrophotometrically by monitoring the increase in absorbance at 240 nm (Barnby et
22 al., 1990). The experiment was done in duplicates for each yeast strain.

23 **3. Results and Discussion**

1 Beside *K. marxianus* CCT 3172 isolated from cocoa and reported to have strong PG
2 activity (Schwan and Rose, 1994), *P. anomala* S16 and *P. kluyveri* S13Y4 were found
3 to exhibit the strongest extracellular PG activity on agar plates of the polygalacturonic
4 acid medium with diameters of clearing zones around colonies of 29-32 mm (Fig 1).
5 The two strains of *H. uvarum* and the remaining strains of *P. anomala* and *P. kluyveri*
6 showed significantly weaker PG activity with diameters of clearing zones around
7 colonies of 7.0-11 mm.

8 Secretion of pectinolytic enzymes by yeasts was also investigated in YPA and CB
9 media. In YPA broth, all yeasts were found to secrete PG (Table 1) but no pectin lyase
10 or pectin esterase was found to be produced by the yeasts examined. It has been
11 reported that the most common enzyme found to be secreted by pectinolytic yeasts is
12 PG (Federici, 1985; Barnby et al., 1990; Mckay, 1990; Blanco et al., 1994; Schwan et
13 al, 1997). However, in few cases, other pectinolytic enzymes were detected in yeasts
14 such as pectin esterase and pectin lyase, secreted by a strain of *S. cerevisiae* isolated
15 from wine (Gainvors et al., 1994) and pectin esterase secreted by *Rhodotorula* spp.
16 associated with softening of olives (Vaughn et al., 1969).

17 In the present study, the highest amounts of PG were found to be secreted by *K.*
18 *marxianus* CCT 3172, *P. anomala* S16 and *P. kluyveri* S13Y4, while other yeast strains
19 produced scarce amounts of the enzyme, which agrees with screening of PG activity,
20 determined by the plate method for hydrolysis of polygalacturonic acid (Fig 1).
21 Furthermore, the PG was found to be secreted in higher amounts when *P. anomala* S16
22 and *P. kluyveri* S13Y4 were grown in CB than when grown in PYA broth (Table 1). It
23 appears that CB is a good substrate for production of PG by the two strains of *P.*
24 *anomala* and *P. kluyveri*. When yeast strains were grown in YPA without addition of

1 glucose, they showed scarce growth and no PG activity was detected in yeasts free
2 supernatant (results not shown), which indicates that the investigated yeasts were unable
3 to utilize polygalacturonic acid as a sole carbon source. This is in accordance with the
4 findings of Sanchez et al. (1984), who found that the pectinolytic yeasts isolated from
5 cocoa were unable to grow or to produce PG in a pectin medium not supplemented with
6 glucose. In addition, when yeasts were grown in YNB, which only contained glucose
7 but no polygalacturonic acid, PG activity was not detected in the yeasts free supernatant
8 (results not shown). Same observation for two strains of *S. cerevisiae*, NCYC 365 and
9 NCYC 73 were reported by McKay (1990), who suggested that this might be due to the
10 absence of enzyme induction during growth on glucose without polygalacturonic acid.
11 In addition to polysaccharides and sucrose, it has been reported that in beans of arabica
12 coffee the concentrations of glucose and fructose at the end of maturation and at the
13 time of picking of cherries i.e. before fermentation were about 0.03 and 0.04 % dry
14 weight, respectively (Rogers et al., 1999). The presence of monosaccharides in coffee
15 seems to be a prerequisite for secretion of polygalacturonase during fermentation by the
16 pectinolytic yeasts.

17 The effect of growing of *P. anomala* S16, *P. kluyveri* S13Y4 and *K. marxianus* CCT
18 3172 in YPA broth adjusted to different pH values in the range of 3.0 to 8.0 on secretion
19 of polygalacturonase is shown in Fig 2a. It was found that the yeasts investigated
20 showed an optimum pH of 6.0 for enzyme secretion. The effect of incubation
21 temperature for the yeasts to secrete polygalacturonase is shown in Fig 2b. Increasing
22 temperature from 15 to 40 °C for the three yeasts showed a maximum at 30 °C followed
23 by a sharper decrease in enzyme secretion. From other study, it has been reported that
24 the optimum pH and temperature for production of polygalacturonase by

1 *Kluyveromyces wickerhammi* were in a range of 3.8-4.5 and 28.5-35.5 °C, respectively
2 (Moyo et al., 2003). The difference in the optimum pH for PG production from our
3 investigations might be due to the different yeast species studied.

4 The stability of the PG produced by the three yeasts was investigated in different
5 buffers at pH values from 3.0 to 8.0 as shown in Fig 3a. Optimum pH for the enzyme
6 activity produced by the reference strain *K. marxianus* CCT 3172 and *P. anomala* S16
7 was 5.5; while PG produced by *P. kluyveri* S13Y4 was found to have a maximum
8 activity at pH 5.0. Furthermore, the enzyme activity was examined at different
9 temperatures in the range of 10-70 °C. For *K. marxianus* CCT 3172 and *P. anomala*
10 S16, the maximum PG activity was observed at 40 °C (Fig 3b). The optimal temperature
11 for activity of PG produced by *P. kluyveri* S13Y4 was 50 °C. For the three yeasts, PG
12 activity decreased rapidly above 50 °C and the enzyme was inactive at 70 °C. Vaughn et
13 al. (1969) reported that the optimum pH and temperature for PG produced by
14 *Rhodotorula* spp. were 6.0 and 50 °C, respectively. The PGs produced by *Trulopsis*
15 *candida*, *Candida norvegensis*, *Kluyveromyces fragilis* and *Saccharomyces chevalieri*
16 were found to have the same optimum pH of 5.0, however different optimum
17 temperatures for the PGs produced by the four yeasts were obtained (Sanchez et al.,
18 1984). In another study, two optimum pH values of 4.5 and 5.0 were reported for the
19 PG activity produced by two strains of *S. cerevisiae*; on the other hand, an optimum
20 temperature of 45 °C was reported for both strains (Blanco et al., 1997). It appears that
21 the optimum pH and temperatures for PGs produced by yeasts might vary between yeast
22 species and also between strains within the same species. From this study and previous
23 studies, it can also be observed that the optimum pH of PGs produced by yeasts is
24 within the acidic region. During coffee fermentation, the initial pH in fresh pulped

1 cherries is reported to be between 5.5 and 6.0 (Wootton, 1963), which after 20 to 25 h
2 of fermentation is reduced to 3.5 (Avallone et al, 2001). A final pH of 4.3 after 36 h of
3 coffee fermentation has also been recorded (Van Pee and Castelein, 1972). The pH
4 values reported during fermentation are within the range of pH at which the PGs
5 secreted by the investigated yeasts are active, which indicates that the yeasts in the
6 present study originating from coffee fermentation, could have a role in mucilage
7 degradation. Contrary, *Klebsiella pneumoniae* and *Erwinia hericola*, which have been
8 isolated from coffee fermentation, were found to secrete pectin lyase with optimum pH
9 of 8.5, which is far from the acidic coffee fermentation conditions i.e. pH 5.3-3.5
10 (Avallone et al., 2002). Sakiyama et al. (2001) also found a pectin lyase with an
11 optimum pH of 7.9 to be secreted by *Paenibacillus amylolyticus*; a bacterium isolated
12 from arabica coffee cherries.

13 From the present study, it can be seen that only PG was produced by the investigated
14 yeasts. Significant differences in the amounts of PG secreted were found between the
15 yeast species as well as between strains of the same species. The extracellular PG
16 produced by *P. anomala* S16 and *P. kluyveri* S13Y4 have an optimum activity at pH of
17 5.5 and 5.0 respectively, which is within the range of pH conditions that occur during
18 coffee fermentation. In addition to their ability to secrete PG, *P. anomala* S16 and *P.*
19 *kluyveri* S13Y4 were among the yeasts, which have been found to inhibit growth and
20 ochratoxin A (OTA) production by *Aspergillus ochraceus* (Masoud and Kalsoft, 2005;
21 Masoud et al., 2005). Therefore, the strong pectinolytic strains of *P. anomala* and *P.*
22 *kluyveri* appear to have potential to be used as starter cultures for mucilage degradation
23 and biological control against OTA producing fungi during coffee fermentation.
24 Further studies of the PG produced by these yeasts are needed and the ability of the

1 investigated yeasts to degrade mucilage *in vivo* i.e. during coffee processing shall be
2 conducted, which will be accompanied by evaluation of the quality of coffee beans *i.e.*
3 appearance, colour and aroma.

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Legends to figures

Figure 1. Screening for pectinolytic activity of six strains of *Pichia anomala* (S12, S13, S14, S15, S16, S17), four strains of *Pichia kluyveri* (S4Y3, S7Y1, S8Y4, S13Y4), two strains of *Hanseniaspora uvarum* (S3Y8, S15Y2) and the reference strain *Kluyveromyces marxianus* CCT 3172 grown on yeast polygalacturonic acid medium (YPA). Pectinolytic activity was determined by the diameter of the clearing zone around yeasts colonies. Bars represent standard deviations.

Figure 2. Polygalacturonase (PG) production by *P. anomala* S16, *P. kluyveri* S13Y4 and reference strain *K. marxianus* CCT 3172 when grown in yeast polygalacturonic medium (YPA) adjusted to different pH (a) and incubated at different temperatures (b). Bars represent standard deviations.

Figure 3. Polygalacturonase (PG) activity of *P. anomala* S16, *P. kluyveri* S13Y4 and reference strain *K. marxianus* CCT 3172 when the enzyme reaction mixtures were incubated in phosphate buffers of pH 3.0 to 8.0 (a) or when the enzyme reaction mixtures were incubated at temperatures of 10 to 70 °C (b). Bars represent standard deviations.

Table 1. Production of polygalacturonase (PG) by six strains of *Pichia anomala* (S12, S13, S14, S15, S16, S17), *P. kluyveri* (S4Y3, S7Y1, S8Y4, S13Y4), two strains of *Hanseniaspora uvarum* (S3Y8, S15Y2) and *Kluyveromyces marxianus* CCT 3172 grown in yeast polygalacturonic acid medium (YPA) and in coffee broth (CB).

Yeasts	PG activity ($\mu\text{mol galaturonic acid /min}$)	
	YPA \pm SD ¹	CB \pm SD
S12	5.5 \pm 0.3	5.2 \pm 0.4
S13	5.3 \pm 0.3	4.7 \pm 0.5
S14	4.5 \pm 0.6	5.0 \pm 0.3
S15	5.1 \pm 0.3	5.5 \pm 0.6
S16	20.0 \pm 0.7	46.0 \pm 0.4
S17	5.7 \pm 0.7	5.3 \pm 0.6
S4Y3	7.2 \pm 0.4	5.1 \pm 0.7
S7Y1	5.3 \pm 0.7	5.8 \pm 0.3
S8Y4	6.1 \pm 0.3	5.2 \pm 0.5
S13Y4	17.0 \pm 0.7	36.5 \pm 0.5
S3Y8	3.4 \pm 0.8	3.5 \pm 0.7
S15Y2	2.7 \pm 0.4	3.2 \pm 0.8
CCT3172 ²	24.5 \pm 0.3	16.6 \pm 0.6

¹ Standard deviation for two trials.

² Reference strain *Kluyveromyces marxianus* isolated from cocoa (Schwan and Rose 1994).

Fig 1

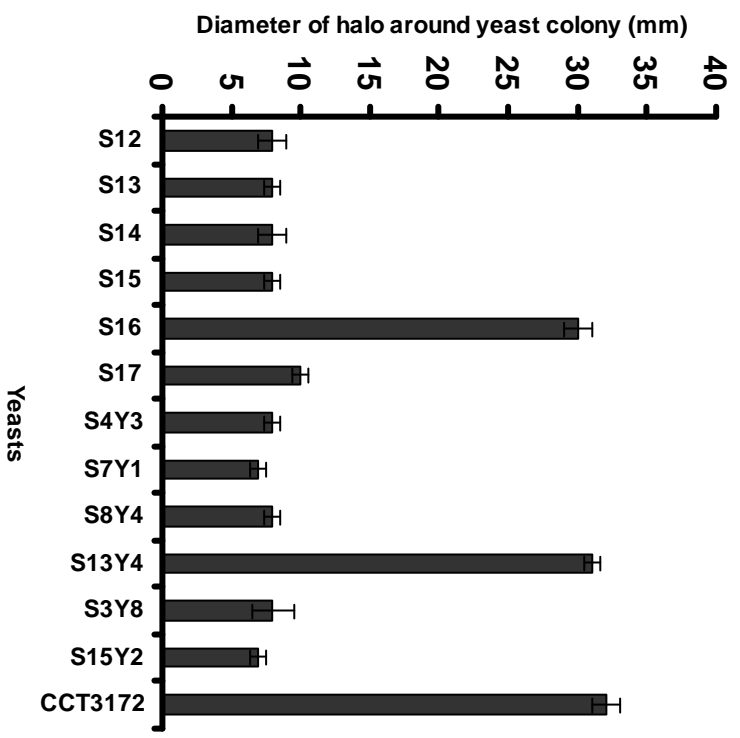


Fig 2a

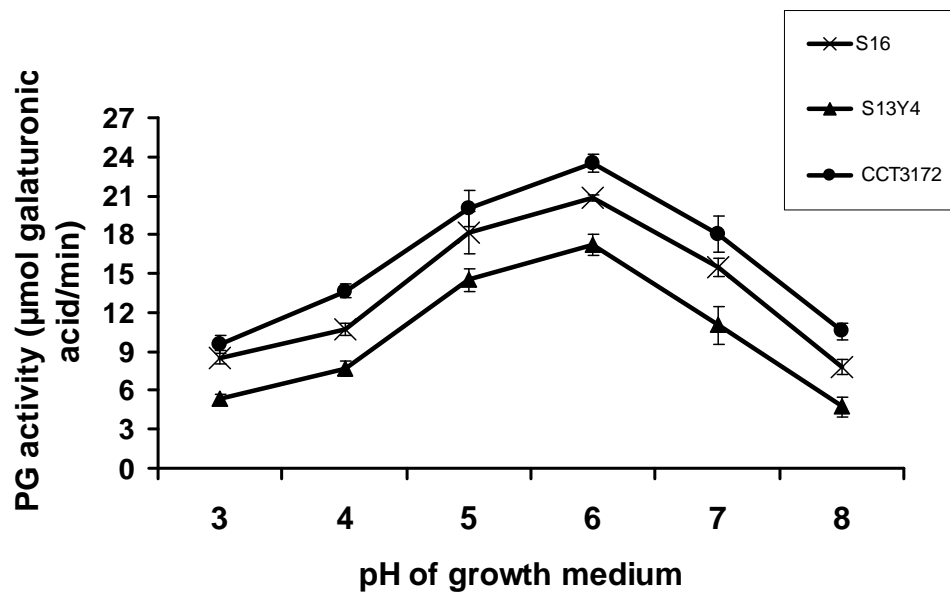


Fig 2b

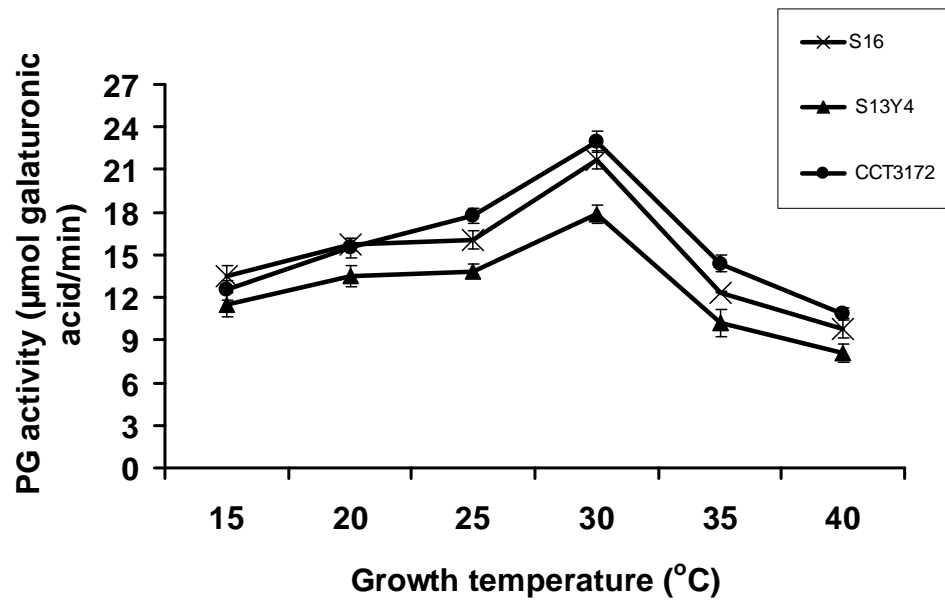


Fig 3a

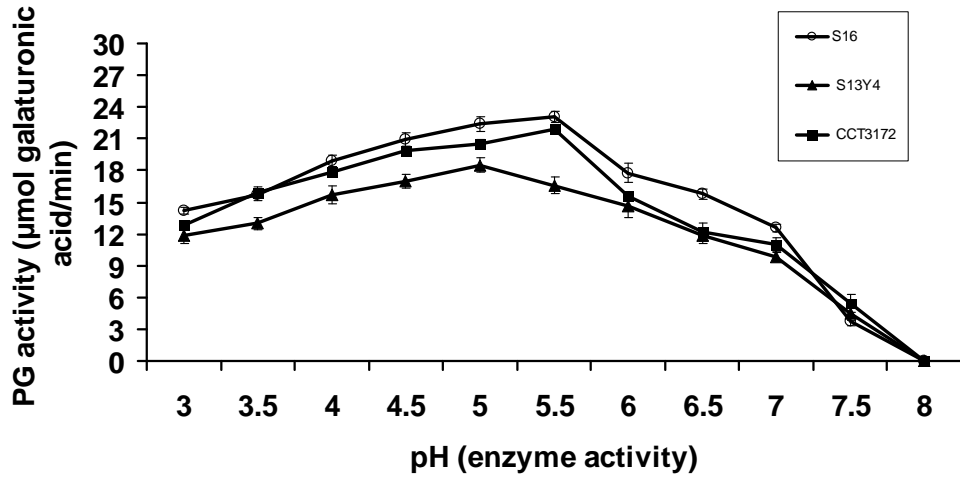


Fig 3b

