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

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**Abstract**

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

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

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

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

## 1. Introduction

Coffee is mainly grown in tropical and subtropical regions but is consumed worldwide. A large number of species of the genus *Coffea* have been identified. The commercial coffee beans belong to the two species *Coffea arabica* and *Coffea canephora* var. *robusta*. To separate beans from pulp coffee is processed by dry or wet method. The dry method is mainly used for *robusta* coffee, which has a thin pulp that allows direct drying (Fowler et al., 1998). In general, cherries of *Coffea arabica* worldwide are processed by the wet method. However, more than 80 % of arabica coffee in Brazil, Yemen and Ethiopia are processed by the dry method (Brando, 2004). In wet processing of coffee, the ripe coffee cherries are pulped followed by fermentation and drying (Fowler et al., 1998).

It is a main aim of coffee fermentation to remove the pectineous mucilage adhering to coffee beans. According to studies that have been carried out, disagreements about which microoganisms is responsible for pectin degradation exist. Vaughn et al. (1958) examined dry and semi-dry processed Brazilian coffee and found mainly Gram-negative bacteria with pectinolytic activity belonging to the genera *Aerobacter* and *Escherichia*. In addition, pectinolytic species of *Bacillus* and a variety of pectinolytic filamentous fungi were isolated. From wet processed coffee in Hawaii, species of *Erwinia*, *Escherichia* and most commonly *Erwinia dissolvens*, were isolated to which degradation of mucilage was related (Frank et al., 1965). On the other hand, *Kluyveromyces marxianus, Saccharomyces bayanus, Saccharomyces cerevisiae* var. *ellipsoideus,* and *Schizosaccharomyces* species, which were found to have pectinolytic activity, were isolated from fermented robusta coffee in India (Agate and Bhat, 1966).

Pectin is a complex heteropolysaccharide composed of D-galacturonic acid residues joined by α-1.4-linkages, which form homogalacturonan chains (Be Miller, 1986). Enzymes that act on pectin molecules include the pectinesterases (PE) and the depolymerases. PE are able to de-esterify pectin by hydrolysis of the methyl ester group, while depolymerases split the main chain (Be Miller, 1986). The depolymerases are divided into polygalacturonases (PG), which cleave the glycosidic bonds by hydrolysis, and lyases (PL), which break the glycosidic bonds by β-elimination at esterified D-galacturonic acid units. Pectinolytic enzymes from yeasts are mainly endo-PG; they have been reported in *Rhodotorula* spp. (Vaughn et al., 1969), *Cryptococcus albidus* (Federici, 1985), *K. marxianus* (Lim et al., 1980; Barnby et al., 1990; Schwan and Rose, 1994; Schwan et al., 1997), *S. cerevisiae* (Blanco et al., 1994), and several species of *Candida* (Call et al., 1985; Sanchez et al., 1984; Stratilova et al., 1998).

The aim of the present work was to study the ability of *P. anomala*, *P. kluyveri* and *H. uvarum* predominant during wet processing of arabica coffee in Tanzania (Masoud et al., 2004) to produce pectinolytic enzymes in a laboratory substrate as well as in coffee broth. Furthermore, the effects of pH and incubation temperature on the production of polygalacturonase and its activity were also investigated.

## 2. Materials and methods

### 2.1 Cultures

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

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

**2.2 Screening for polygalacturonase activity**

The yeasts were propagated in 25 ml of malt yeast glucose peptone (MYGP) broth [3.0 g yeast extract (Difco, Detroit, MI, USA), 3.0 g malt extract (Difco), 5.0 g bactopeptone (Difco), 10.0 g glucose (Merck, Darmstadt, Germany) per 1 liter distilled water] at 25 $^{o}$C for 48 h. After propagation cells were harvested by centrifugation at 3000 x g for 10 min, and resuspended in diluent saline peptone (SPO) [0.1 % (w/v) bactopeptone (Difco), 0.85 % (w/v) NaCl (Merck), 0.03 % (w/v) $Na_2H_2PO_4$, $2H_2O$ (Merck), adjusted with 1.0 M NaOH (Merck) and 1.0 M HCl (Merck) to pH 5.6]. Cell concentrations were estimated by microscopy using a counting chamber (Neubauer) and the suspensions were diluted to final concentrations of $10^6$ cells/ml. Screening for pectinolytic activity was modified from a procedure described by Zink and Chatterjee (1985). Three spots of 10 µl of each yeast suspension were placed on plates of polygalacturonic acid specific medium [7.0 g yeast nitrogen base (YNB) (Difco), 5.0 g glucose (Merck), 5.0 g polygalacturonic acid (Sigma), 20 g agar (Difco) per 1 liter distilled water]. The plates were incubated for 48 h at 25 $^{o}$C and then flooded with 6.0 M HCl (Merck), where clear halo around yeast colonies indicated pectinolytic activity. The experiment was done in triplicates.

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

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

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

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

### 2.3.1 The effects of pH and incubation temperature on production of polygalacturonase by yeasts

Yeasts were propagated in YPA medium at pH values of 3.0-8.0 and incubated at temperatures of 15-40 $^{o}$C for 48 h. Production of PG by yeasts was determined as described above.

### 2.3.2 The effects of pH and temperature on polygalacturonase activity

PG activity was determined at different pH values by incubation of the reaction mixture described above with different phosphate buffers pH 3.0-8.0. Furthermore, the effect of incubation temperature of the reaction mixture on PG activity was investigated at 10-70 $^{o}$C.

### 2.4 Assay for pectin esterase activity

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

### 2. 5 Assay for pectin lyase activity

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

## 3. Results and Discussion

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

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

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

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

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

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

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

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

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

investigated yeasts to degrade mucilage *in vivo* i.e. during coffee processing shall be conducted, which will be accompanied by evaluation of the quality of coffee beans *i.e.* 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 $^{o}$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 (µmol galaturonic acid /min)** | |
|---|---|---|
| | **YPA** ± SD[1] | **CB** ± SD |
| S12 | 5.5 ± 0.3 | 5.2 ± 0.4 |
| S13 | 5.3 ± 0.3 | 4.7 ± 0.5 |
| S14 | 4.5 ± 0.6 | 5.0 ± 0.3 |
| S15 | 5.1 ± 0.3 | 5.5 ± 0.6 |
| S16 | 20.0 ± 0.7 | 46.0 ± 0.4 |
| S17 | 5.7 ± 0.7 | 5.3 ± 0.6 |
| S4Y3 | 7.2 ± 0.4 | 5.1 ± 0.7 |
| S7Y1 | 5.3 ± 0.7 | 5.8 ± 0.3 |
| S8Y4 | 6.1 ± 0.3 | 5.2 ± 0.5 |
| S13Y4 | 17.0 ± 0.7 | 36.5 ± 0.5 |
| S3Y8 | 3.4 ± 0.8 | 3.5 ± 0.7 |
| S15Y2 | 2.7 ± 0.4 | 3.2 ± 0.8 |
| CCT3172[2] | 24.5 ± 0.3 | 16.6 ± 0.6 |

[1] Standard deviation for two trials.

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

**Fig 1**

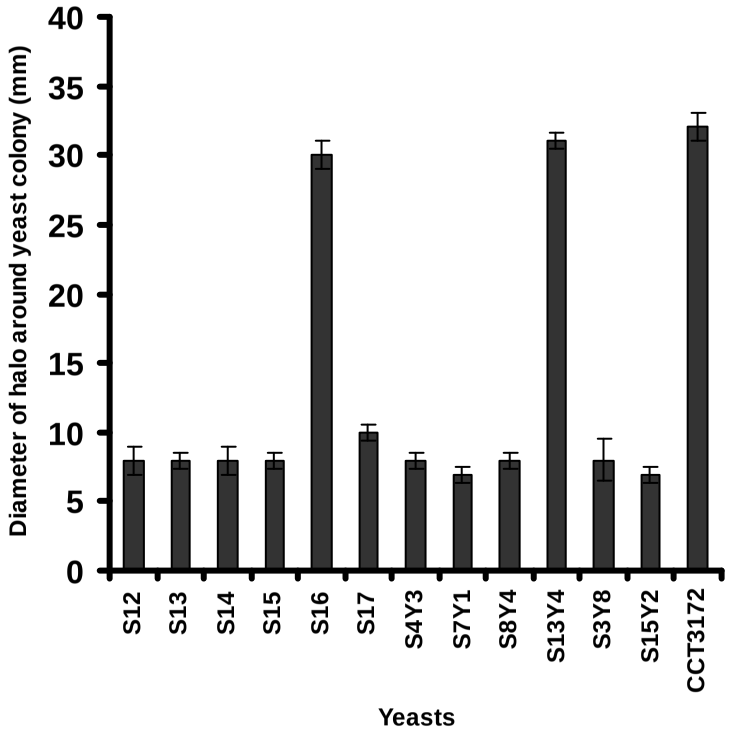

Diameter of halo around yeast colony (mm)
40
35
30
25
20
15
10
5
0
S12
S13
S14
S15
S16
S17
S4Y3
S7Y1
S8Y4
S13Y4
S3Y8
S15Y2
CCT3172
Yeasts

**Fig 2a**

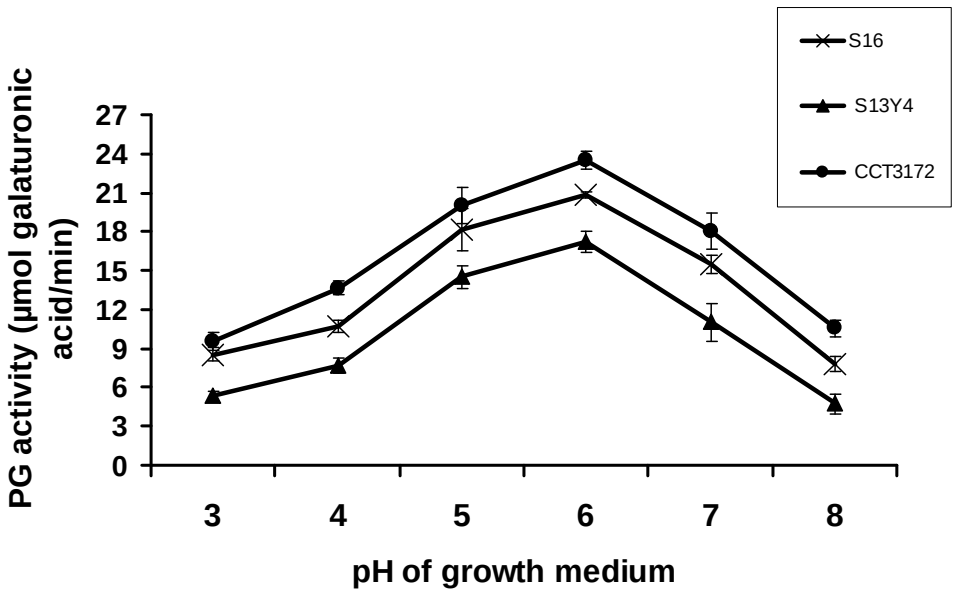

S16
S13Y4
CCT3172
PG activity (µmol galaturonic acid/min)
27
24
21
18
15
12
9
6
3
0
3
4
5
6
7
8
pH of growth medium

**Fig 2b**

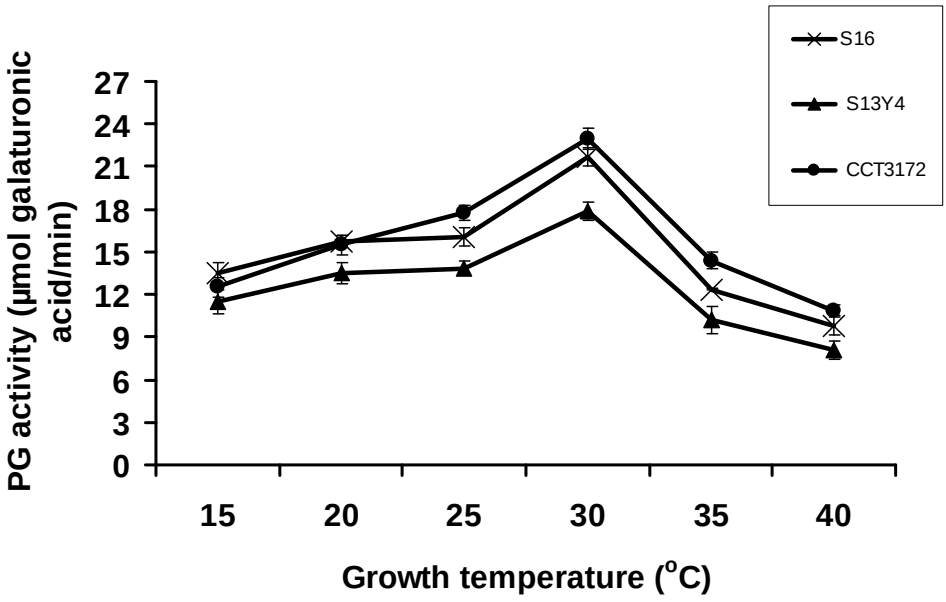

S16
S13Y4
CCT3172
PG activity (µmol galaturonic acid/min)
27
24
21
18
15
12
9
6
3
0
15
20
25
30
35
40
Growth temperature (°C)

**Fig 3a**

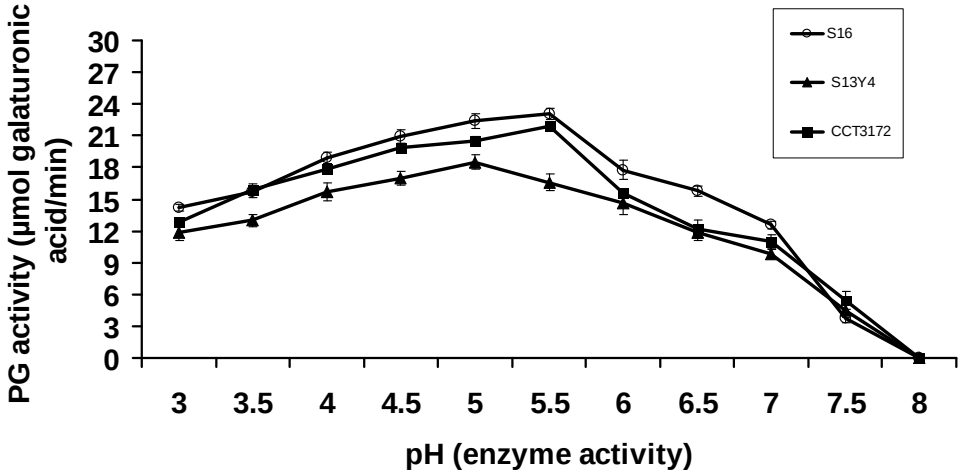
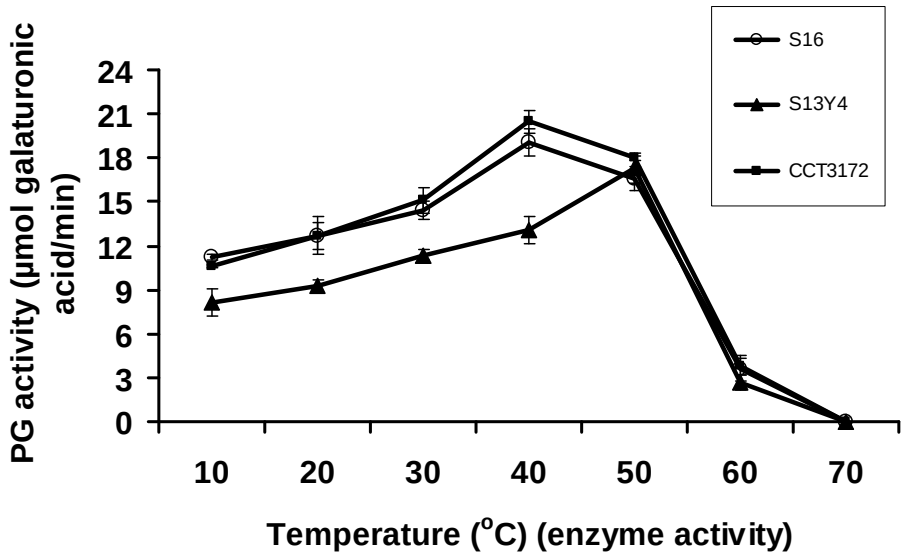

PG activity (µmol galaturonic acid/min)
30
27
24
21
18
15
12
9
6
3
0
3
3.5
4
4.5
5
5.5
6
6.5
7
7.5
8
pH (enzyme activity)
S16
S13Y4
CCT3172

**Fig 3b**


PG activity (µmol galaturonic acid/min)
24
21
18
15
12
9
6
3
0
10
20
30
40
50
60
70
Temperature (°C) (enzyme activity)
S16
S13Y4
CCT3172