

Isolation and Screening of Polygalacturonase-Producing *Bacillus* sp.

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ABSTRACT

Polygalacturonases (PGases) are the depolymerizing enzymes which cleave the α (1,4) glycosidic bonds between two galacturonic acid residues and are being used for the pretreatment of waste water from vegetable food processing industries containing pectinaceous material, processing and degumming of plant fibers such as ramie, sunn hemp, bael, etc. In the present study, 20 bacterial strains were isolated from different samples: soil, spoiled vegetables and fruits, and Godavari River water. All the strains were screened for their ability to utilize pectin as the sole carbon source. Out of the 20 bacterial strains studied, 12 possessed pectinolytic activity as evident from zones of hydrolysis on pectin plates. Selected strains were used for pectinase production by submerged fermentation using 0.25% citrus pectin and the enzyme filtrates were assayed for PGase activity by the DNS method. The effect of temperature, pH on enzyme activity and different media on enzyme production was studied. Maximum enzyme production was shown by strain 11 with medium 1 and optimum PGase activity was found to be at pH 9 at 60°C. Based on cultural and morphological studies the strains were preliminarily identified and assigned to the genus *Bacillus*.

Keywords: *Bacillus*, pectin, PGase, zone of hydrolysis

Abbreviations: DNS, dinitro salicylic acid; NCIM, National Collection of Industrial Microorganisms; PGase, polygalacturonase; PGA, polygalacturonic acid; SmF, submerged fermentation; YEPC, yeast extract pectin calcium chloride

INTRODUCTION

Pectic substances are high molecular weight, negatively charged, acidic polysaccharides that are present as the major components of the middle lamella of plants in the form of calcium pectate and magnesium pectate (Jayani *et al.* 2005; Murad *et al.* 2011). Pectins are polymers of D-galacturonic acid residues linked by α (1, 4) glycosidic linkages with a small number of L-rhamnose, arabinose, galactose and xylose as the side chains (Gummadi and Kumar 2005; Shembekar *et al.* 2009). The enzymes hydrolyzing these pectic substances are broadly classified as pectinases and include polygalacturonases (PGases; EC 3.2.1.15), pectin esterases, pectin lyases and pectate lyases depending upon their mode of action (Hoondal *et al.* 2002; Kar *et al.* 2011). PGases catalyze the hydrolysis of α (1,4) glycosidic linkages in polygalacturonic acid (Kashyap *et al.* 2001; Murad *et al.* 2011). Pectinases are produced by plants (Prasanna *et al.* 2006) and microorganisms such as bacteria (Kapoor *et al.* 2001), fungi (Fahmy *et al.* 2008) and a few yeasts (Serrat *et al.* 2002). Microbial pectinases are extensively used in fruit juice processing, vegetable oil extraction, processing and degumming of plant fibres, processing of alcoholic beverages, treatment of industrial waste water, paper and pulp industries and have also been reported to be used for the purification of viruses (Favela-Torres *et al.* 2006; Arunachalam *et al.* 2010; Zeni *et al.* 2011). In the present study, an attempt was made to identify bacterial strains with pectinolytic activities from soil, water and decomposed vegetable samples with the objective of detecting superior strains.

MATERIALS AND METHODS

Microorganisms

Pectinolytic bacteria were isolated (Sunnotel and Nigam 2002) from soil, water and decomposed vegetables using YEPC broth

(pH 7.0) containing 1% yeast extract, 0.25% pectin and 0.05% CaCl_2 by enrichment culturing and incubated at 37°C for 48 h. The broth was streaked on YEPC agar plates and morphologically distinct colonies were further subcultured onto fresh nutrient agar slants. Pure cultures were maintained by repeated subculture onto fresh slants and were stored at 4°C.

Screening of pectinolytic bacteria by plate assay

Isolated cultures were spot inoculated onto the same YEPC medium with 2% agar and incubated at 37°C for 48 h. To detect the zone of hydrolysis, the plates were flooded with Grams iodine. *Bacillus firmus* (NCIM-2462) was used as the control to compare the zone of hydrolysis of the isolated strains. The genus of the isolated strains was identified by morphological and biochemical characterization following "The Prokaryotes" II edition (Balows *et al.* 1991).

Pectin, a polysaccharide, when broken down into simple sugars by the enzymatic action of PGase, appears yellow-orange while pectin alone becomes brown after the reaction with Grams iodine.

Production of polygalacturonases by submerged fermentation

The strains showing large and clear zones were used for enzyme production. 20 ml of YEPC medium in 100-ml conical flasks were inoculated with 5% inoculum of overnight cultures grown in nutrient broth and incubated at 37°C for 48 h at 150 rpm. The samples were centrifuged at 10,000 rpm for 15 min at 4°C. The clear supernatant was collected and used for the assay of PGase activity.

Assay of polygalacturonase activity

The reaction mixture containing 0.1 ml of 1% PGA (Sigma Aldrich Pvt. Ltd) and 0.1 ml of supernatant was incubated at 40°C for 10 min. PGase activity was determined by measuring the

Table 1 Morphological and biochemical characters of all the isolates.

Test	1	8	9	10	11	12
Gram staining	+	+	+	+	+	+
Endospore	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
Voges-Proskauer	+	+	+	+	+	+
Growth in anaerobic agar	+	+	+	+	+	+
Growth at 50°C	+	+	+	+	+	+
Growth in 7% NaCl	+	+	+	+	+	+
Acid and gas from glucose	-	-	-	-	-	-
NO ₃ reduced to NO ₂	+	+	+	+	+	+
Starch hydrolysis	+	+	+	+	+	+
Growth at 65°C	-	-	-	-	-	-
Rods 1 m wide	-	-	-	-	-	-
pH in VP medium <6	-	-	-	-	-	-
Acid from glucose	+	+	+	+	+	+
Hydrolysis of casein	-	-	-	-	-	+
Parasporal bodies	-	-	-	-	-	-

**Fig. 1** Pectin (0.25%) plate showing zones of hydrolysis by the strains.

amount of reducing groups released using the DNS method (Miller 1959). One unit of enzyme activity was defined as 1 μ mol of galacturonic acid released per min under the given conditions.

Effect of pH and temperature on enzyme activity

The effect of pH on the enzyme activity was determined by using different buffers (citrate-phosphate, sodium acetate, Tris-HCl and carbonate/bicarbonate) at pH values ranging from 3.8 to 11. The effect of temperature on enzyme activity was determined at 5°C increments from 30 to 80°C.

Effect of different media on enzyme production

Four different media were used to maximize the production of PGases. They were YEPC medium (HiMedia, Mumbai) (1% yeast extract, 0.25% citrus pectin, 0.05% CaCl₂, pH 7.2; Sunnotel and Nigam 2002), enriched medium 1 (1% pectin, 0.5% yeast extract, 0.1% KH₂PO₄, 0.01% MgSO₄·7H₂O, pH 7.2; Kapoor *et al.* 2000), enriched medium 2 (1% pectin, 0.14% (NH₄)₂SO₄, 0.6% K₂HPO₄,

0.20% KH₂PO₄, 0.01% MgSO₄·7H₂O, pH 7.2; Soares *et al.* 1999), enriched medium 3 [0.5% pectin, 0.2% K₂HPO₄, 0.25% MgSO₄·7H₂O, 0.5% Na₂HPO₄, 0.1% yeast extract, 0.179% casein, 2 ml/L micronutrient solution (in 1 L: 0.1 g Al(OH)₃, 0.5 g SnCl₂·2H₂O, 0.05 g KI, 0.05 g LiCl, 0.8 g MnSO₄·2H₂O, 0.5 g H₃BO₃, 0.1 g ZnSO₄, 0.1 g NiSO₄, 0.05 g BaCl₂, 0.05 g (NH₄)₆Mo₇O₂₄·4H₂O; pH 7.2; Sharma and Satyanarayana 2006].

RESULTS AND DISCUSSION

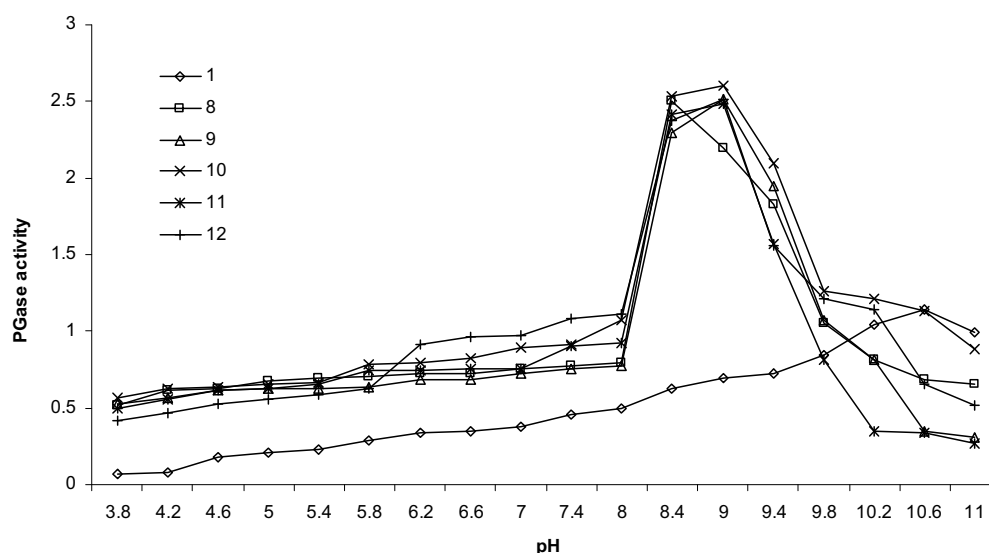
Selection of strains with pectinolytic activity

Out of 20 bacterial strains isolated 12 strains showed a zone of hydrolysis on YEPC agar plates. Of the 12 strains, six (1, 8, 9, 10, 11 and 12) showed a maximum zone of clearance and were selected for enzyme production (**Fig. 1**). Identification of these six strains was done by morphological and biochemical characterization. The results showed that they are Gram positive, endospore-forming and Catalase-positive rod-shaped bacteria belonging to the genus *Bacillus* (**Table 1**).

Effect of pH and temperature on polygalacturonase activity

Maximum PGase activity among all the strains range from pH 8.0 to 9.0 (2.59 U/ml/min) except for strain 1, which had an optimum pH range between 10 and 11 (1.15 U/ml/min) (**Fig. 2**). The optimum temperature for strains 1, 8, 10 and 11 was 60°C (5.03 U/ml/min) while that for strains 9 and 12 was 45°C (3.01 U/ml/min) (**Fig. 3**).

Singh *et al.* (2010) reported maximum PGase production at pH 6.8 and 30°C (6.2 U/ml/min) using *Bacillus sphaericus* (MTCC 7542). Further, Sharma and Satyanarayana (2006) found maximum PGase production at pH 10.5 and 50°C using *Bacillus pumilus*. Odeniyi *et al.* (2009) found that at pH 6.0 and 50-60°C, pectinase production was maximum using *Bacillus coagulans*. Other pectinases having optimum pH in an alkaline range have been reported from different *Bacillus* spp. such as NT-2, NT-6, NT-33 and NT-82 (Cao *et al.* 1992). Kapoor *et al.* (2002) reported maximum PGase activity at an optimum temperature of 60°C (pH 10.0) from *Bacillus* sp. MG-cp-2. PGases are mostly active under alkaline conditions, which is commercially important in the treatment of industrial waste water-containing pectinaceous material and also useful in processing and degumming of bast fibers (Horikoshi 1990; Kapoor *et al.* 2000). However, only a few reports are available on the applications of alkaline pectinases.

**Fig. 2** Effect of pH on polygalacturonase activity (U/ml/min) of the isolates.

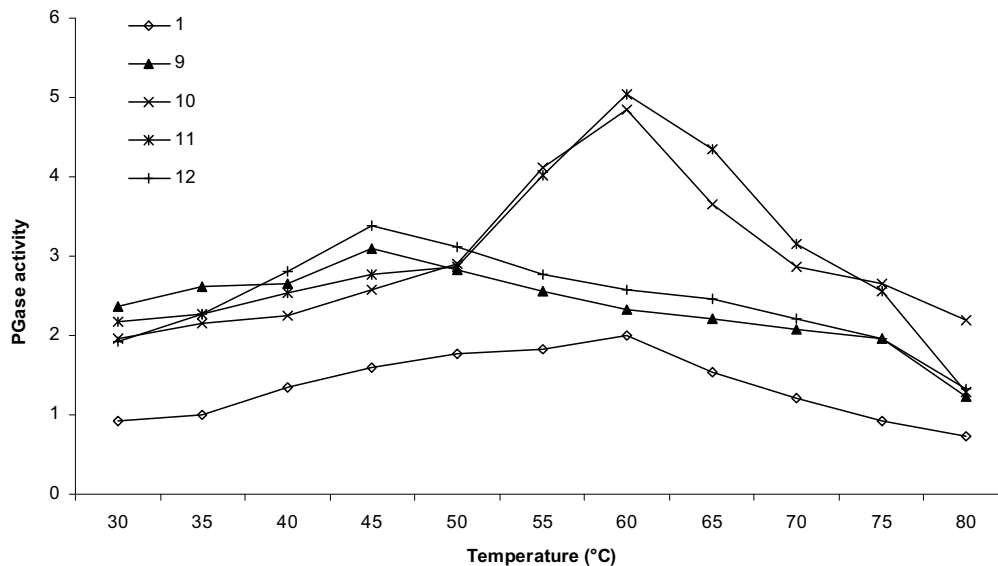


Fig. 3 Effect of temperature on polygalacturonase activity (U/ml/min) of the isolates.

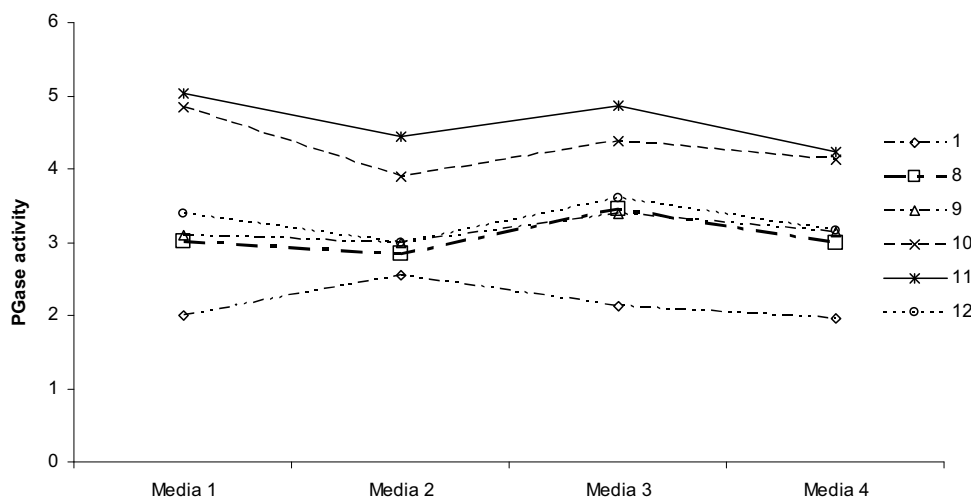


Fig. 4 Effect of different media on production of polygalacturonase (U/ml/min) by the isolates.

Screening of different media for production of PGase

Maximum PGase activity found in strains 8, 9 and 12 when grown in medium 3 was 3.45, 3.39 and 3.6 U/ml/min, respectively. Further, strains 10 and 11 showed highest PGase production (4.85 and 5.03 U/ml/min) in medium 1 (Fig. 4). However, there was no significant PGase production in medium 4 by any of the strains. When the concentration of pectin was increased in the medium, all strains showed a decrease in the production of PGase. Kashyap *et al.* (2000) also reported maximum production with 1% yeast extract and 0.25% pectin in medium using *Bacillus* sp. DT7 while Singh *et al.* (2010) reported maximum PGase production with 1.25% pectin using *Bacillus sphaericus*.

CONCLUSIONS

Six bacterial strains were isolated and identified as the *Bacillus* genus. Maximum PGase activity was obtained with strain 11 (5.03 U/ml/min) at pH 9, 60°C when medium 1 was used for production. Further improvement of enzyme activity by employing strain development strategies is being considered.

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