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Purification and Characterization of Polygalacturonase using Isolated *Bacillus subtilis* C4

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ABSTRACT

The present investigation is envisaged to study the production of alkaline and thermostable polygalacturonase using isolated soil *Bacillus subtilis* (C4) by submerged fermentation. The extracted enzyme was subjected to partial purification which included ammonium sulphate precipitation, dialysis, ion exchange chromatography and affinity chromatography. The cell free supernatant polygalacturonase activity was found to be in the 30-40% salt saturation fraction. Specific activity of polygalacturonase increased from 33.68 to 93.05 U mL^{-1}. Further the fraction was loaded onto anion exchanger, DEAE Sephacel equilibrated with 20mM Tris HCl (pH 7.5 buffer). The proteins were eluted with NaCl gradient. 9.84 fold purification of the enzyme was achieved and its specific activity was found to be 915.6 U mL^{-1}. When subjected to affinity chromatography the purification fold increased up to 15 fold with specific activity 13789.11 U mL^{-1}. The homogeneity of the polygalacturonase was judged by SDS-PAGE. It was found that PGs had a molecular weight ranging between 43 and 66 kDa. The partially purified enzyme was characterized; the enzyme was stable at 60°C up to 60 min at pH 9.0. The enzyme was showing 100% substrate specificity to polygalacturonic acid. A slight activation effect was observed in the presence of Ca^{2+} and Mg^{2+}.

Key words: Polygalacturonase, *Bacillus subtilis,* fermentation, purification, characterization

INTRODUCTION

Enzymes are proteins that catalyze the chemical reaction both biosynthetic and degradative occurring in living cells. Pectic substances are high molecular mass glycosidic macromolecules found in higher plants. They are present in the primary cell wall and are the major components of the middle lamellae, a thin extracellular adhesive layer formed between the walls of adjacent young cells. In short, they are largely responsible for the structural integrity and cohesion of plant tissues (Rombouts and Pilnik, 1980). Pectinases are an enzyme group that catalyzes pectin substance degradation through depolymerization (hydrolases and lyases) and deesterification (esterases) reactions. One of the most important and widely used commercial pectinase is polygalacturonase. Polygalacturonase catalyse hydrolysis of α-1, 4-glycosidic linkage in poly galacturonic acid producing D-galacturonate (Coutinho and Henrissat, 1999) Pectinolytic enzymes are naturally produced by many organisms like bacteria, fungi, yeasts, nematodes, protozoan and plants. Alkaline pectinases are generally produced by bacteria particularly species of *Bacillus,* but are also made by some filamentous fungi and yeasts (Kashyap *et al*., 2000) and acidic pectinases are

produced by fungi. Submerged fermentation and solid state fermentation have been successfully used in pectinase production by fungi (Dinu *et al.*, 2007) and bacteria (Jacob *et al.*, 2008). Pectinase production occupies about 10% of the overall manufacturing of enzyme preparations. These enzymes are widely used in the food industry in the production of juices, fruit drinks and wines (Semenova *et al.*, 2003).

They may be used in the pretreatment of waste water from vegetable food processing that contains pectin residues, the processing of textile fibers such as flax, jute and hemp, coffee and tea fermentation, vegetable-oil extraction and the treatment of paper pulp (Kashayp *et al.*, 2001).

The analysis of enzyme activity in the crude extract does not indicate either an isolated action or the presence of a multienzymic system working in synergy on the substrate degradation. The characterization of purified enzymes is an important research line since it provides discrimination between the complex components about substrate degradation mechanism activity conditions and enzyme synthesis regulation.

MATERIALS AND METHODS

Microorganisms: *Streptomyces lydicus* (MTCC 4067), *Bacillus pumilus* (MTCC 1456), *Aspergillus niger* (MTCC 281), *Bacillus subtilis* C4 (Isolated) were used in this study. First three cultures were procured from Microbial Type Culture Collection, MTCC), Chandigarh, India and the fourth one was isolated from the soil, identified, maintained in nutrient agar and subcultured monthly. All the chemicals and reagents used were of analytical grade and obtained from reputed chemical manufacturers. The isolated pure cultures were screened for extracellular polygalacturonase using agar media containing pectin as a substrate (plate assay). Three replicates were prepared for each sample.

Polygalacturonase production by submerged fermentation: Erlenmayer flasks (250 mL) containing media composed of 2 g of KH_2PO_4, 1 g of $(NH_4)_2 SO_4$, 1 g of $MgSO_4$, 0.95 g of Na_2HPO_4 2 H_2O, 1 g of yeast extract and 5 g of pectin were taken in 100 mL of distilled water. The medium was inoculated with 1 mL of a spore suspension of isolated *Bacillus subtilis* and cultivated at 50°C for 48 h. After 2 days of incubation the medium was centrifuged at 5000 rpm for 15 min and the supernatant was taken for the assay of amount of galacturonic acid produced.

Polygalacturonase assay: Polygalacturonase activity was assayed by the colorimetric method of Miller. 0.5 mL of supernatant was incubated with 1.0% w/v of pectin at 40°C for 10 min under static conditions. After adding 3 mL of di nitro salicylic acid, the mixture was boiled for 15 min and finally diluted to 5 mL with distilled water. The absorbance was measured at 540 nm (Miller, 1959). One unit (U) of polygalacturonase was defined as 1 μmole of galacturonic acid released per mL-min.

Purification of polygalacturonase: The 150 mL of cell free supernatant was fractionated by precipitation with ammonium sulphate between 30-90% of saturation. The saturated solution was left overnight at 4°C, centrifuged at 12,000 rpm for 20 min, dissolved in minimal amount of 10 mM Tris HCl buffer at pH 8 and dialyzed against the same buffer for 24 h at 4°C (Green and Hughes, 1995). The enzyme was purified further in an anion exchanger by loading 3.0 mL sample in a glass column packed with DEAE Sephacel. The column was equilibrated with Tris HCl buffer (20 mM, pH 7.5) and was loaded onto it. The column was washed with Tris HCl buffer containing 20, 30, 40, 50, 100 and 150 mM NaCl concentration. The protein content of the fractions was measured spectrophotometrically at 280 nm and the polygalacturonase

activity was assayed by the method described earlier. The fraction showing polygalacturonase activity was pooled, concentrated and saved for further analysis (Nadaroglu *et al*., 2010).

Affinity chromatography of polygalacturonase with alginate: The ion exchanged enzyme solution was incubated with 0.4 mL of alginate solution (stock solution of 2%, w/v) and the final volume was made up to 4 mL with 0.05 M acetate buffer, pH 5.0. The pH of the reaction mixture was adjusted to 3.8 with 3 M acetic acid and incubated at 25°C for 1 h. The polymer-enzyme complex was precipitated by addition of 0.4 mL of 1 M $CaCl_2$ (final concentration of $CaCl_2$ in the solution was 0.1 M). The precipitate was separated by centrifugation (10,000 rpm for 10 min) and washed thrice with 4 mL of 0.05 M acetate buffer, pH 5.0, containing 0.1 M $CaCl_2$. The difference in the total enzyme activity loaded and the activity present in the supernatant and washings represented the amount of enzyme bound to the polymer. The bound enzyme was recovered by dissolving the complex in 6 mL of 0.05 M acetate buffer, pH 5.0 containing 1 M NaCl and incubated at 4°C for 18 h. Thereafter, the salts were removed by passing the elute through column (Mondal *et al*., 2004).

Characterization of polygalacturonase

Molecular weight determination: Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in 3 mm slab gel of 6% acrylamide in Tris borate buffer at pH 7.1 containing 0.1% SDS using 5% (w/v) stacking and 10% (w/v) separating gels. The gels were stained with Coomassie brilliant blue R-250 and destained by keeping in destaining solution (Stagemann, 1979). Then the gel can be viewed under the transilluminator. The standard protein marker supplied by Bangalore Genei was used for comparing the MW of enzyme sample.

Effect of temperature and pH on enzyme activity: Optimum temperature was evaluated by incubating the enzyme preparation having 0.1mL of enzyme and 0.1 mL of substrate at different temperatures in the range of 10-90°C for 30 min and assayed as described. The effect of pH on activity of enzyme was determined by incubating the reaction mixture using suitable buffers in a final pH range of 3 to 10. The mixtures were incubated at 50°C for 30 min and the enzyme activity was assayed as described.

Effect of substrate specificity on polygalacturonase: The effect of substrate specificity on polygalacturonase enzyme was determined by treating 0.1 mL of crude sample with different substrates like polygalacturonic acid, pectin, amylopectin and potato dextrose agar of concentration 0.1% w/v and its specificity for the substrate was tested.

Effect of enzyme stability: The effect of enzyme stability under optimized temperature and optimized pH was studied at various time intervals ranging 30, 60, 90,120, 150 and 180 min.

Effect of chelating agents: The effect of $HgCl_2$, $ZnCl_2$, $MnCl_2$, $CaCl_2$ and $MgCl_2$ was studied by adding 10 mM concentration each of the above metal to the enzyme mixture, incubated and assayed.

RESULTS AND DISCUSSION

Isolation of culture having polygalacturonase potential: A total of 66 colonies were isolated from four soil samples, on the basis of macroscopic characters and microscopic observations,

eliminating those that appeared close to each other. All 66 isolated colonies were tested for their polygalacturonase potential. Of them, only eight bacterial strains shown polygalacturonase potential. Of the 8 positive isolates, C4 was showing relatively high activity (Fig. 1).

Purification of polygalacturonase: The purification steps for polygalacturonase were summarized in Table 2. Polygalacturonase was partially purified to homogeneity using various steps. Initially, the enzyme was purified by addition of solid ammonium sulphate. The cell free supernatant polygalacturonase activity was found to be in the 30-40% salt saturation fraction (Table 1). Specific activity of polygalacturonase increased from 33.68 to 93.05 U mL^{-1}. Studies have shown that polygalacturonase can be precipitated between 0-90% of ammonium sulphate depending on the source of the enzyme (Buga *et al*., 2010).

Fig. 1: Plate assay of isolate C4 showing clear zone

Table 1: Ammonium sulphate precipitation

Ammonium sulphate saturation (%)	Specific activity (Units mg^{-1})	Purification (fold)
30	93.05	1.00
40	50.49	0.54
50	20.74	0.22
60	15.70	0.16
70	4.30	0.04
80	0.00	0.00

Table 2: Purification profile of Polygalacturonase from isolated *Bacillus subtilis* C4

Step	Collected volume (mL)	Total protein (mg)	Specific activity (Units mg^{-1})	Purification (fold)
Crude	150	182.8	33.68	1.00
Ammonium sulphate	80	16.88	93.05	2.76
Ion exchange	40	2.84	915.60	9.84
Affinity chromatography	14	0.56	13789.11	15.06

Further, the fraction was loaded onto anion exchanger, DEAE Sephacel equilibrated with 20 mM Tris HCl buffer (pH 7.5). The ion exchange elution profile was studied with various concentration gradients of NaCl. The profile revealed one active peak at 40 mM concentration gradient. 9.84 fold purification of the enzyme was achieved and its specific activity was found to be 915.6 U mL^{-1}. When subjected to affinity chromatography the purification fold increased upto 15 fold with specific activity 13789.11 U mL^{-1}.

Characterization of partially purified polygalacturonase

Molecular weight determination by SDS-PAGE electrophoresis: Ion exchange chromatographic fractions were subjected for SDS PAGE analysis. Electrophoresis was carried out on a 6% polyacrylamide containing 0.1% SDS. The molecular weight of the polygalacturonase was determined by comparison of the migration distances of standard marker proteins. It was found that polygalacturonase had a molecular weight ranging between 43 and 66 kDa (Fig. 2).

These findings were similar to other works whose enzyme was purified 280 fold and the overall yield was 8.4%. The specific activity towards PGA was 540 mg of protein. The MW of the enzyme was estimated to be approximately 45,000 daltons by SDS PAGE (Kashayp *et al*., 2001). Similar findings reported the molecular weight of the partially purified polygalacturonase to be 40 and 38 kDa (Buga *et al*., 2010).

Electrophoresis was carried out on a 6% polyacrylamide containing 0.1% SDS. The gel was stained with Coomassie blue R-250 Lane a included the following standard proteins: 1. Myosin, Rabbit Muscle (MW 205 kDa), 2. Phosphorylase (MW 97 kda), 3. Bovine serum albumin (MW 67 kDa), 4. Ovalbumin (MW 43 kDa), 5. Carbonic anhydrase (MW 31kDa):

- Lane b indicates ammonium sulphate fractionation
- Lane c indicates ion exchange chromatographic fraction
- Lane d indicates affinity chromatographic fraction

Effect of pH and temperature on activity of polygalacturonase: The effect of temperature on the exo polygalacturonase was studied at the temperature ranges 10-90°C. The activity of enzyme increased gradually from 20°C and reached maximum at 60°C thereafter, it decreased

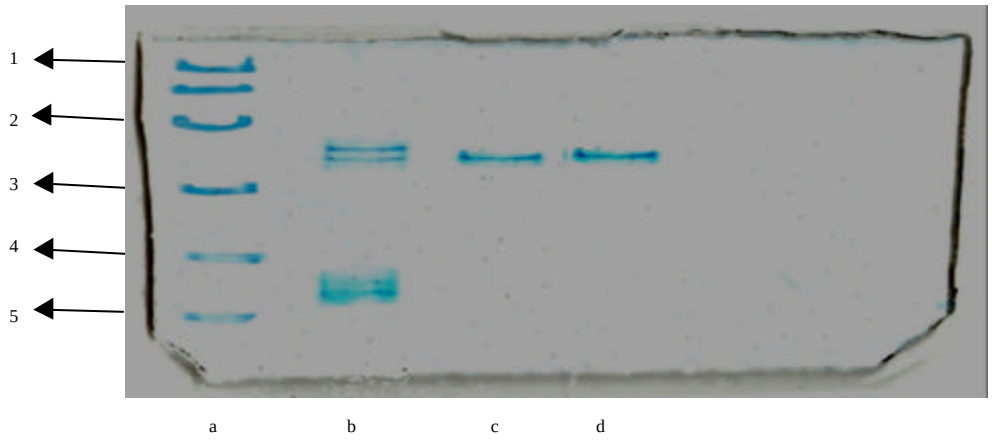

Fig. 2: SDS-polyacrylamide gel electrophoresis of polygalacturonase from *Bacillus subtilis* C4

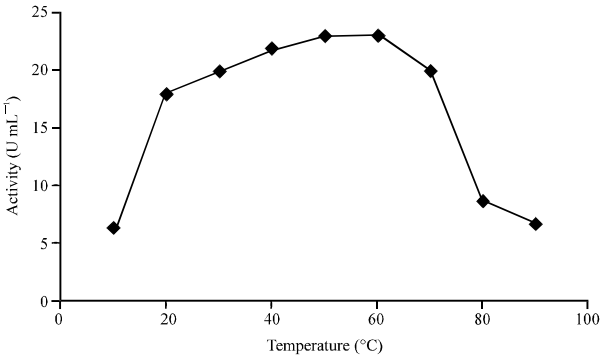

Fig. 3: Effect of temperature on activity of polygalacturonase

Table 3: Effect of pH on activity of polygalacturonase

pH	Activity (U mL^{-1})
3	9.70
4	19.80
5	20.95
6	22.55
7	22.55
8	23.21
9	26.49
10	25.70

(Fig. 3). This was predicted corresponding to increase of kinetic energy. The increasing kinetic energy can lead to increasing of collisions between enzyme and substrates to form a complex of enzyme substrates and finally can increase the product. However at temperatures above 70°C pectinase activity decreased and it was predicted due to partial denaturation of pectinase. This can produce conformational change in the enzyme and as an effect, it becomes difficult for the substrate to enter the active site and enzymatic reaction did not occur easily (Roosdiana *et al*., 2013).

Similar reports have been observed with *Aspergillus flavus* MTCC 7589 (50°C) (Fogarty and Kelly, 1983) *Bacillus* sp. KSM-P576 (55°C) (Kobayashi *et al*., 1999) and *Bacillus* sp. KSM-P7 (60-65°C) (Nakamura *et al*., 1971). Similar findings were observed with *Bacillus* sp. DT7 where polygalacturonase was maximally active at moderately high temperature ranging from 40-60°C with highest activity detected at 60°C (Kashyap *et al*., 2000).

The effect of pH on the exopolygalacturonase was examined at varied pH ranges from 3-10. The activity of the purified enzyme increased gradually from pH 4.0 and reached maximum at pH 9.0 and thereafter it dropped (Table 3).

Pectinase from *Bacillus* sp. DT7 was maximally stable under alkaline conditions of pH 7.5-8.5. Further the enzyme retained 50% of its activity at neutral pH (Mondal *et al*., 2004). Similar reports were in the optimal pH range of 8.9-9.4 for pectinase of *Bacillus polymyxa*. and pH 9.0 was reported for *B. stearothermophilus* (Nagel and Vaughn, 1961; Fogarty and Kelly, 1983) and pH 10.0 was reported for *Bacillus* sp. RK9.

Effect of substrate specificity on activity of polygalacturonase: Effect of various substrates was tested for hydrolysis of galacturonic acid. Of all the substrates examined, only polygalacturonic

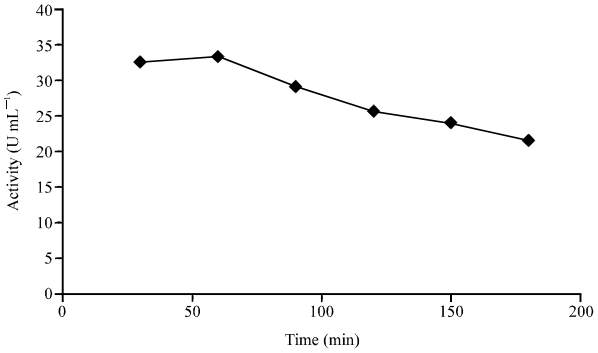

Fig. 4: Effect of enzyme stability at 60°C and at pH 9.0

Table 4: Effect of substrate specificity on polygalacturonase

Substrate (0.1% w/v)	Activity (U mL^{-1})	Relative activity (%)
Polygalacturonic acid	27.95	100
Pectin	26.67	95.42
Amylopectin	17.59	62.93
Potato dextrose agar	12.52	44.79

Table 5: Effect of chelating agents

Chelating ion (10 mM)	Activity (U mL^{-1})
$HgCl_2$	32.59
$CaCl_2$	36.37
$MgCl_2$	33.05
$MnCl_2$	30.60
$ZnCl_2$	23.95

acid was found to be hydrolysed by enzyme and the rate of hydrolysis was found to be 100%. The enzyme catalyses the hydrolysis of pectin, amylopectin and potato dextrose agar at the rate of 95.42, 62.93 and 44.79%, respectively (Table 4). The difference in the rate of hydrolysis suggest that hydrolysis occurred at the same active site of the enzyme molecule with relatively low specificity for the configuration the substrate molecule and kind of bonding which it hydrolysis (Nakamura *et al*., 1971).

Effect of enzyme stability: The effect of enzyme stability at 60°C and at pH 9.0 was studied at various time intervals ranging 30, 60, 90, 120, 150 and 180 min and the enzyme activity was assayed. The purified enzyme was stable upto 60 min at specified conditions and thereafter the activity gradually decreased (Fig. 4). The thermal inactivation of enzymes is always due to denaturation of enzyme proteins (Martins *et al*., 2002).

Similar finding was reported that purified enzyme appeared to be stable and retained its full activity after 1 h incubation period from 40-50°C but the activity was reduced to 20% after 1 h at 60°C (Nadaroglu *et al*., 2010).

Effect of chelating agents: The effect of chelating agents on the enzyme stability was studied (Table 5). A slight activation effect was observed in the presence of Ca^{2+} and Mg^{2+}. Hg^{2+} and Mn^{2+}

also stimulated the PG activity but less than Ca^{2+}. Zn^{2+} was showing partial inhibitory effect. This discrepancy in the divalent metal ion preference suggested that the enzymes might have differential flexibility in the active site (Beg and Gupta, 2003).

Similar results were observed where pectin lyase from *Bacillus pumilus* was inhibited by 10 mM of Hg^{2+} and Mn^{+2} and activated by Ca^{2+}, L-cysteine and ascorbic acid. Metal ions like Ca^{2+} and Mg^{2+} might play a vital role in maintaining the active confirmation of alkaline endo PGs to stimulate the activity (Li *et al*., 2008).

CONCLUSION

Microbial pectinases have tremendous potential in commercial applications and acidophilic pectinases have extensive application in extraction and clarification of juice and wine. However work on the utilization of alkaline pectinases remains underdeveloped as only few reports are available on application of this enzyme. Alkaline pectinases have been used in several conventional industrial processes, such as textile and plant fiber processing, coffee and tea fermentation, oil extraction and treatment of industrial waste water containing pectinacious material. With increased understanding and knowledge of the mechanism of pectin-degrading microorganisms and their enzymes, alkaline pectinases have made their way into several other biotechnological processes such as purification of plant viruses and paper making, most of which, despite sound interesting, have yet to be commercialized.

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