

Purification and Characterization of Polygalacturonase Produced by *Aspergillus niger* AN07 in Solid State Fermentation

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Received: Oct 18, 2016; Revised: Jan 28, 2017; Accepted: Feb 04, 2017

Abstract

Polygalacturonase, an industrial enzyme has been produced from various fungal isolates using solid state and submerged fermentation techniques. The challenge has been yield, extraction and cost of production. In the present study, a low cost solid substrate, dried papaya peel was employed for polygalacturonase production using *Aspergillus niger* AN07. Polygalacturonase enzyme from *Aspergillus niger* AN07 was purified to 24.8 fold with a 52.6% recovery through anion exchange chromatography on DEAE-cellulose and gel filtration chromatography using Sephadex G-200. The SDS-PAGE revealed that the enzyme was monomeric with a molecular weight of 64.5 kDa. The optimum pH and temperature were 5.0 and 55°C, respectively. This enzyme was stable over a wide pH range (4.0–7.0) and relatively high temperature of 55°C for 1 h. The *K_m* and *V_{max}* values of polygalacturonase for polygalacturonic acid were 2.6 mg/l and 181.8 µmol/ml/min, respectively. The purified enzyme could digest the polygalacturonic acid into oligosaccharides with a small amount of galacturonic acid as visualized on thin layer chromatography.

Keywords: Polygalacturonase, Purification, Characterization, DEAE-cellulose chromatography, Sephadex G-200 chromatography

Introduction

Pectin, a major constituent of plant cell wall is mainly composed of galacturonic acid residues. The enzymes that hydrolyze pectic substances are broadly known as pectinolytic enzymes, which include pectin methyl-esterase, polygalacturonase (exo-polygalacturonase and endo-polygalacturonase) and pectin lyase on the basis of their mode of action [1, 2]. Polygalacturonase is one of the most widely distributed enzymes in bacteria [3], fungi [4] and plants [5]. Polygalacturonase (pectinase; EC. 3.2.1.15) hydrolyzes the α-1, 4-glycosidic bond between galacturonic acid residues and releases oligomers of D-galacturonic acid.

Polygalacturonase has a share of 25% in the global sales of food enzymes and has numerous biotechnological applications such as in fruit juice extraction and its clarification, scouring of cotton, degumming of plant fibers, waste water treatment, vegetable oil extraction, tea and coffee fermentation, bleaching of paper, in poultry feed as additive and in alcoholic beverages [6-9].

Polygalacturonase has been purified and characterized from various fungal isolates viz. *Mucor flavus* [2], *M. circinelloides* [10], *A. niger* [11-13], *A. awamori* [14, 15], *A. carbonarius* [16], *Rhizopus oryzae*

[17], *Sclerotium rolfsii* [18], *Penicillium sp.* [19, 20], *Bispora sp.* [21] and *Neosartorya fischeri* [22].

Polygalacturonase production from microorganisms has been reported under both submerged (SmF) and solid-state fermentation (SSF) conditions [23]. SSF holds tremendous potential for the production of industrial enzymes. Agro-industrial residues are generally considered as suitable substrates for the production of enzymes in SSF [24-26].

In the present study, polygalacturonase produced by locally isolated *A. niger* AN07 in SSF has been purified using ammonium sulfate precipitation, ion exchange and gel filtration chromatographies. The purified enzyme has been characterized for its physico-chemical properties.

Materials and Methods

Materials

Pectin, polygalacturonic acid and D-galacturonic acid were purchased from Sigma-Aldrich Chemical Co., USA. Microbial substrates, papaya peel and orange peel were collected from local juice shops and dried before use. Other chemicals used in the study were of analytical grade.

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Fungal isolate and Inoculum preparation

Samples were collected from the soil of fruit processing sites at Indore, India. A fungal strain capable of producing polygalacturonase was screened on potato dextrose agar (PDA) medium containing pectin. The medium consisted of (g/l) 200 g potato extract; 20 g dextrose; 10 g of citrus pectin; 25 g agar; pH 5.6. The isolated fungal strain was identified on the basis of morphological, microscopic and molecular characteristics as *A. niger* AN07.

Inoculum of *A. niger* AN07 was prepared on PDA slant incubated at 30°C for 4 days. The spores formed were scrapped using sterile glass rod, filtered through sterile glass wool and counted using counting chamber. Spore suspension of 1×10^6 spores/ml prepared in sterile distilled water was used for inoculation in SSF medium.

Solid state fermentation

Polygalacturonase production in SSF was carried out using dried papaya peel and orange peel in the ratio 2:1 (w/w). For SSF, 10 g of papaya peel having particle size 2 mm was taken in 250 ml Erlenmeyer flask and initial moisture content 90% (v/w) was maintained with distilled water. The substrate was sterilized in an autoclave at 121°C for 20 min at 15 psi and made to undergo SSF for 6 days at 30°C by inoculating with 1 ml of spore suspension having spore content 1×10^6 spore/ml (Unpublished results).

Enzyme assay and Protein estimation

Enzyme was extracted from the solid fermented medium using 0.1 M sodium acetate buffer, pH 5.0 in the ratio of 1:10 (w/v). It was homogenized using chilled pestle and mortar and subsequently subjected to incubation at room temperature for 30 min in an orbital shaker (Remi BL- 120) at 100 rpm. The extracted medium was centrifuged at $10,000 \times g$ for 30 min in the cold condition (0 to 4°C) and the supernatant was used as the crude enzyme.

Polygalacturonase was assayed by measuring the release of reducing sugars using Nelson and Somogyi method [27, 28]. The reaction mixture consisted of 1000 μ l of 0.1% (w/v) polygalacturonic acid prepared in 0.1 M acetate buffer, pH 5 and 990 μ l of distilled water. The reaction was started using 10 μ l of the enzyme. The contents were incubated at 55°C for 20 min and galacturonic acid content liberated by the enzyme was determined as reducing sugar equivalent using galacturonic acid as a standard. One unit (U) of the enzyme activity was defined as the amount of enzyme that catalyzes the release of one μ mol of galacturonic acid per min under the conditions of enzyme assay. Enzyme activity has been expressed as units per gram dry substrate (U/gds). Total soluble protein was determined according to the method of Lowry et al. using bovine serum albumin as the standard [29].

Enzyme purification

To the crude enzyme, solid ammonium sulfate was added to get 0 to 30% saturation. The pH of the suspension was kept constant with the help of dilute ammonia. After the addition of ammonium sulfate, suspension was incubated for 3 h in the cold condition to precipitate proteins. Thereafter, it was centrifuged at $10,000 \times g$ for 30 min to collect the pellet and to the supernatant, more ammonium sulfate was added to get 30 to 60% saturation and suspension was incubated for 4 h in the cold condition to precipitate proteins. Thereafter, it was centrifuged at $10,000 \times g$ for 30 min to collect the pellet and to the supernatant, more ammonium sulfate was added to get 60 to 90% saturation and suspension was incubated overnight in the cold

condition to precipitate proteins. The precipitated proteins were collected by centrifugation at $10,000 \times g$ for 30 min in the cold condition. The pellets after each centrifugation were dissolved in 75 mM sodium phosphate buffer, pH 7.0 and tested for polygalacturonase enzyme activity. The 60 to 90% fraction was centrifuged at $10,000 \times g$ for 20 min at 0-4°C to get clear supernatant. The centrifuged enzyme (60 to 90% fraction) was desalted using Sephadex G-25 column.

The desalted enzyme solution was loaded on to a DEAE cellulose column (2 x 15 cm; bed volume 47 ml) equilibrated with 75 mM sodium phosphate buffer, pH 7.0. Unbound proteins were collected by elution with two bed volumes of buffer before starting the gradient and were analyzed for enzyme activity. A 200 ml linear gradient of 0-1 M NaCl, prepared in the same buffer, was used for elution of the enzyme. The active fractions (~20 ml) constituting a single peak were pooled and concentrated by reverse dialysis against solid sucrose using a nitrocellulose membrane (Hi media- 135). For this, the pooled fraction was put in the nitrocellulose membrane and its ends were sealed. Thereafter, it was covered by about 200 g solid sucrose in a beaker and incubated over night in the cold condition to reduce the volume up to nearly 2 ml. The concentrated enzyme was applied onto a Sephadex G-200 column (1.5 x 50 cm; bed volume 88 ml) equilibrated with 75 mM sodium phosphate buffer, pH 7.0. Protein fractions having polygalacturonase activity were pooled and concentrated again by reverse dialysis.

Native PAGE and Zymogram study

The homogeneity of the purified enzyme was estimated using 10% Native PAGE according to the method of Laemmli [30]. Two wells were loaded with enzyme in each half of the polyacrylamide gel. After electrophoresis, first half of gel was incubated in 0.1 M sodium acetate buffer pH 5.0 for 10 min. It was then transferred in 1% (w/v) polygalacturonic acid prepared in 0.1 M sodium acetate buffer pH 5.0 and incubated at 50°C for 30 min. After incubation, the gel was washed gently with distilled water and treated with 1% (w/v) cetyl trimethylammonium bromide (CTAB) which revealed polygalacturonase activity as transparent band on an opaque white background. In the other half of the gel, protein bands were stained using Coomassie brilliant blue 250 (CBB-250).

SDS-PAGE

SDS-PAGE analysis of the purified enzyme was performed as described by Laemmli [30] and the molecular weight of the purified enzyme was determined using Bio-Rad molecular weight marker BL 03 (14-95 kDa). Electrophoresis was carried out in the polyacrylamide gel consisting of a 4% (w/v) stacking gel and 10% (w/v) resolving gel.

Effect of substrate concentration

The K_m and V_{max} values of the purified enzyme were determined with polygalacturonic acid prepared in 0.1 M sodium acetate buffer, pH 5.0 as substrate. To determine the optimal value, different substrate concentrations from 1 to 10 mg/l were used in assay conditions. A double-reciprocal plot for the enzyme was used for estimating K_m and V_{max} values and calculated using Lineweaver-Burk equation:

$$\frac{1}{v} = \frac{K_m}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}}$$

Effect of pH on enzyme activity and pH stability

The effect of pH on the enzyme activity was determined by preparing the reaction mixture in 0.1 M sodium-acetate buffer, pH 4 to 6 with interval of 0.2. In order to determine the pH stability, the enzyme was pre-incubated in 0.1 M sodium-acetate buffer (pH 3.0-6.0), 0.1 M phosphate buffer (pH 7.0, 8.0) and 0.1 M glycine-NaOH buffer (pH 9.0, 10.0) at 4°C for 1 h and then assayed for polygalacturonase activity in 0.1 M sodium-acetate buffer pH 5.0 with polygalacturonic acid as substrate.

Effect of incubation temperature and thermostability

The effect of incubation temperature was performed by incubating the enzyme and substrate reaction mixture at temperatures ranging from 30°C to 70°C with difference of 5°C. Thermal stability was investigated by measuring the activity of the purified enzyme solution after it had been kept for 1 h, in the absence of substrate, at temperatures ranging from 50°C to 65°C with an interval of 5°C. The remaining polygalacturonase activity was determined at optimum pH and temperature, using polygalacturonic acid as a substrate.

Effect of metal ions

The effect of metal ions such as K⁺, Na⁺, Cu²⁺, Ca²⁺, Ba²⁺, Co²⁺, Hg²⁺, Fe²⁺, Ni²⁺, Mg²⁺, Mn²⁺, Zn²⁺, Cr³⁺, Al³⁺ and Pb²⁺ (all chloride salts) on enzyme activity was determined at concentrations of 2.0 mM. The higher concentration, 4 mM and 6 mM were also used for ions which were giving considerable activity at 2 mM concentration.

Product identification

Thin Layer Chromatography was done to assess the product released due to the enzyme action from polygalacturonic acid. 100 µl of purified enzyme mixed with 500 µl of 0.5% polygalacturonic acid was incubated for 0, 10, 20 and 40 min at 55°C. At each time interval, 1.0 µl was drawn and loaded on silica gel plate. The enzyme reaction was terminated immediately by drying the spot with dryer. Samples were run with solvent system containing acetone: butanol: water in ratio of 5:4:1 (v/v) [31]. Thereafter, ethanol containing 5% sulfuric acid was spread over the plate and dried by heating up to 100°C in a dry chamber which resulted in the appearance of dark colored oligosaccharide spots.

Results and Discussion

Purification of polygalacturonase

Purification results are summarized in Table 1. Polygalacturonase produced by *A. niger* AN07 solid-state fermentation was purified to near homogeneity by a combination of anion exchange and gel filtration chromatographic methods. The crude enzyme extract was precipitated by ammonium sulfate saturation and the 60-90% retentate was subjected to anion exchange chromatography where a major peak of polygalacturonase activity was detected (Fig. 1). The unbound enzyme along with most of the contaminating proteins came out in the form of colored fractions. No polygalacturonase activity was determined in these unbound fractions. The enzyme was eluted

Table 1: Purification of polygalacturonase produced by *A. niger* AN07 using SSF.

Purification steps	Enzyme Units	Total Protein (mg)	Specific Activity (U/mg)	Fold Purification	Yield %
Crude extract	2576	365	7.1	-	100
Ammonium Sulphate fraction	2307	187.9	12.3	1.7	89.6
DEAE Cellulose chromatography	1533.8	47.9	32.0	4.5	59.5
Sephadex G-200 chromatography	1354.1	7.7	174.9	24.8	52.6

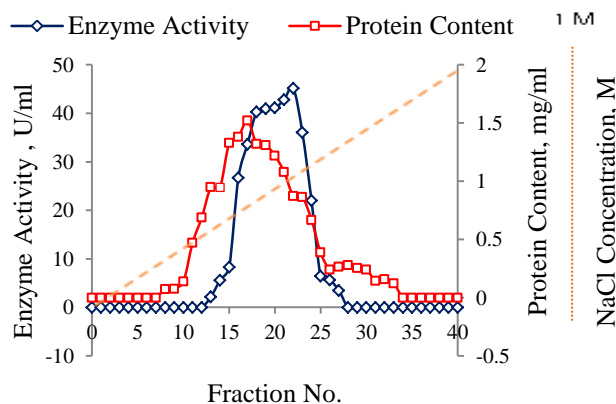


Fig. 1: Elution profile of polygalacturonase from DEAE-cellulose anion exchange chromatography. The enzyme was eluted using a gradient of 0-1.0 M NaCl prepared in 75 mM phosphate buffer, pH 7.0.

out as a single peak at 0.40 M sodium chloride concentration as calculated using the formula given by Morris and Morris [32]. The enzyme fractions were pooled and concentrated by reverse dialysis. The concentrated pool was fractionated separately by gel exclusion chromatography on a Sephadex G-200 column for the final purification step. In this chromatography, single peak of enzyme activity was observed (Fig. 2). Fractions containing polygalacturonase activity were stored at 4°C until use. The yield of purified polygalacturonase was 52.6% with a purification fold of 24.8 and specific activity 174.9 U/mg. It looks that high specific activity is due to the presence of high pectin solid substrate, dried papaya peel during solid state fermentation.

Purification and characterization of polygalacturonase from several fungal sources, such as *A. awamori* [14, 15], *Sclerotium rolfsii* [18], *Penicillium sp.* [20], *Bispora sp.* [21], have been reported. Kant et al. [12] reported 6.5 fold enzyme purification with specific activity 54.3

U/mg and a yield of 5.0%. Polygalacturonase from *A. niger* CFR 305 has been purified using activated charcoal with only 4-fold purification [33]. Therefore, results indicated that specific activity of the enzyme in the present case is higher than the reported studies [12, 33].

Native-PAGE analysis and zymogram studies revealed that the enzyme was purified to homogeneity (Fig. 3). In SDS PAGE study, enzyme molecular mass has been estimated to be 64.5 (± 1.6) kDa on 10% separating gel, when stained by CBB (Fig. 4). Singh and Rao [34] reported two polygalacturonases from *A. niger* namely PG II and PG IV having molecular weight 61 and 38 kDa, respectively. Yuan et al. [20] reported cloning of *Penicillium sp.* polygalacturonase I having molecular mass 37.5 kDa into *Pichia pastoris*. *M. flavus* polygalacturonase molecular weight has been reported to be 40 kDa [2]. Nakkeeran et al. [16] reported 42 kDa molecular weight of *A. carbonarius* polygalacturonase.

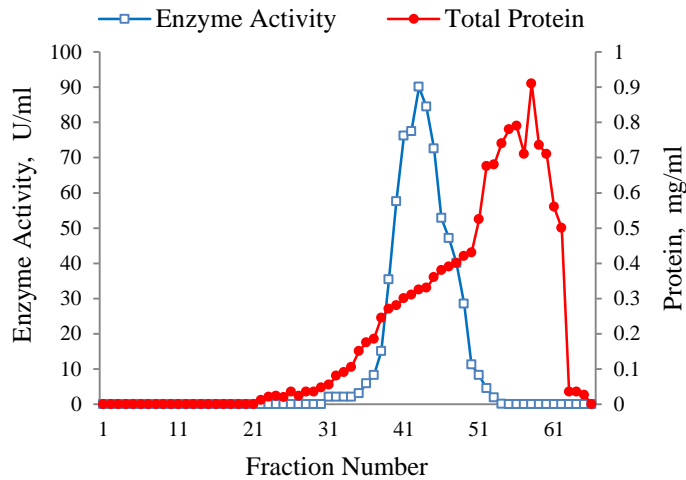


Fig. 2: Elution profile of polygalacturonase on Sephadex G-200.

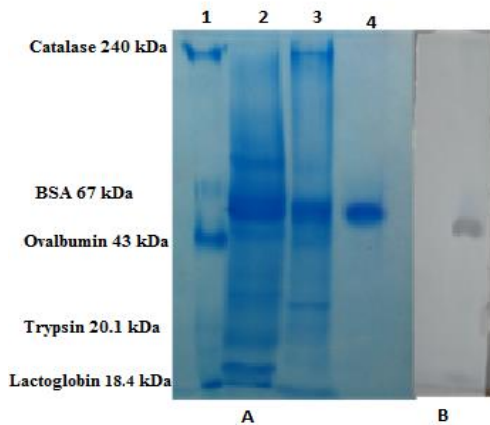


Fig. 3: (A) Native-PAGE analysis of purified polygalacturonase produced from *A. niger* AN07. Lane 1: molecular weight markers; Lane 2: crude enzyme; Lane 3: enzyme purified by Ion Exchange Chromatography; Lane 4: purified enzyme and (B) Zymogram of purified enzyme.

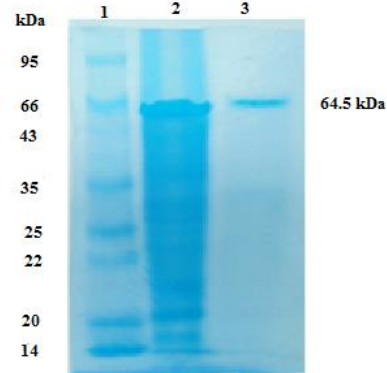


Fig. 4: SDS-PAGE of purified polygalacturonase from *A. niger* AN07. Lane 1: Bio-Rad molecular weight markers; Lane 2: crude Endo-PGase enzyme; Lane 3: purified Endo-PGase enzyme.

Effect of pH and pH stability

The optimum pH of the purified enzyme has been found to be 5.0 (Fig. 5A). However, other reports have suggested alkaline polygalacturonase [7]. The purified enzyme retained 100% activity after pre-incubation at pH 4.0 to 7.0 for 1 h followed by assay at pH 5.0 (Fig. 5B). Polygalacturonase produced from fungus *A. niveus* showed pH stability between 3.0 and 5.0 [35]. Earlier reports have shown the pH stability of polygalacturonases to vary from 2.5 to 12.0 [7].

Effect of temperature and thermostability

The measurement of polygalacturonase activity at different

temperatures showed that the activity increased up to 55°C and then declined progressively (Fig. 6A). The optimum temperature of the purified polygalacturonase is found to be 55°C as the enzyme exhibited maximum activity at this temperature.

The thermostability study of the purified enzyme revealed that it is stable at 55°C for 1 h but further increase in temperature led to the loss of enzyme activity (Fig. 6B). At temperature 60°C and 65°C, the residual activity after 1 h was 59.3 and 6.1%, respectively. The results are in accordance with earlier reports [7, 36] showing optimum enzyme activity in the range of 30-70°C and thermal stability up to 75°C.

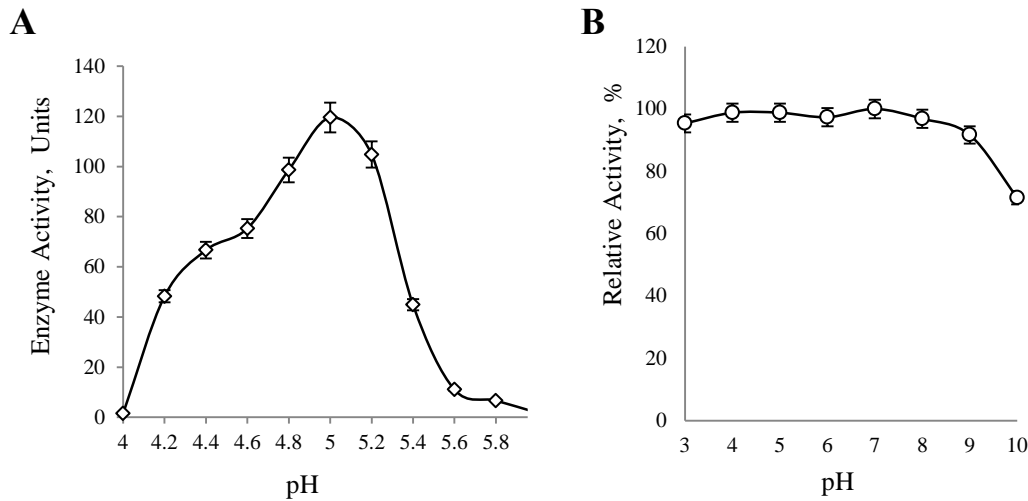


Fig. 5: (A) Effect of pH on polygalacturonase activity, showing maximum activity at pH 5.0 and (B) pH stability of polygalacturonase pre-incubated at different pH at 4°C for 1 h with 100% stability at pH between 4-7.

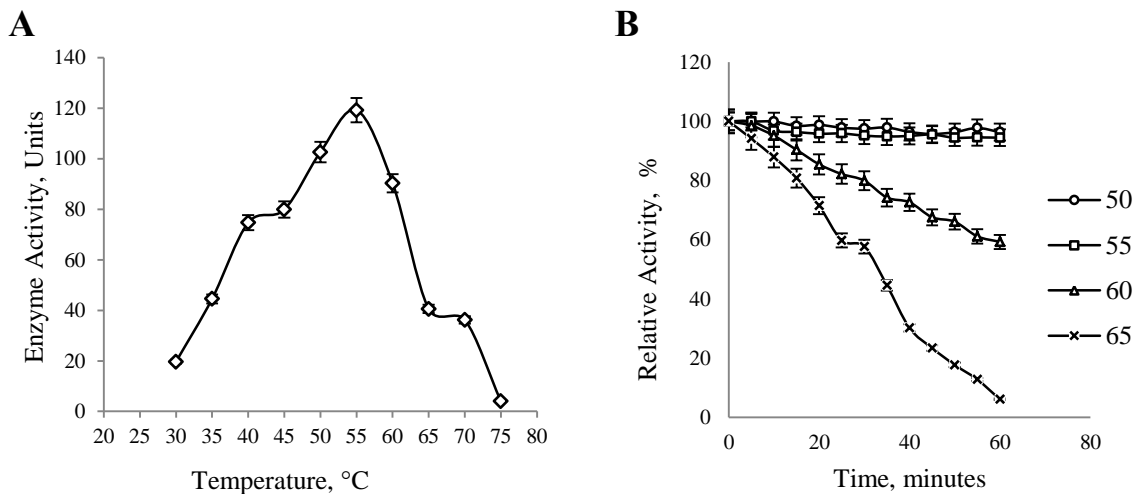


Fig. 6: (A) Effect of temperature on polygalacturonase activity showing optimum activity at 55°C and (B) Thermostability of polygalacturonase produced from *A. niger* AN07. The enzyme showed thermal stability up to 55°C for 1 h.

Determination of K_m and V_{max}

The kinetic constants of the purified polygalacturonase were calculated by fitting the data to a linear regression on Lineweaver-Burk double-reciprocal plot. The K_m and V_{max} values obtained from the Lineweaver-Burk plot were 2.6 mg/l and 181.8 $\mu\text{mol/ml/min}$ showing high affinity of the enzyme polygalacturonase (Fig. 7).

K_m values of the two polygalacturonase enzymes, PG II and PG IV from *A. niger* were found to be 0.12 and 0.72%, respectively [33]. In another report, the K_m of polygalacturonase from *A. niger* was found to be 0.083 mg/ml and V_{max} 18.21 $\mu\text{mol/ml/min}$. Yuan et al. [20] reported K_m and V_{max} of polygalacturonase to be 19.5 mg/ml and 909.1 U/min/mg, respectively.

Effect of metal ions

Among various metal ions, Pb^{2+} , Al^{3+} , Cr^{3+} , Zn^{2+} , Mn^{2+} , Ni^{2+} , Fe^{2+} , Hg^{2+} , Co^{2+} , Ba^{2+} , Ca^{2+} and Cu^{2+} at 2 mM concentration inhibited the

enzyme activity. The effectiveness of metal cations as inhibitors for polygalacturonase was in the order of $\text{Al}^{3+} > \text{Mn}^{2+} > \text{Fe}^{2+} > \text{Zn}^{2+} > \text{Ni}^{2+} > \text{Ba}^{2+} > \text{Cr}^{3+} > \text{Cu}^{2+} > \text{Ca}^{2+} > \text{Co}^{2+} > \text{Hg}^{2+} > \text{Pb}^{2+} > \text{K}^+ > \text{Mg}^{2+}$ with 100%, 99.4%, 99.3%, 98.9%, 98.8%, 98.1%, 97.3%, 95.6%, 95.1%, 93.2%, 87.6%, 78.2%, 24.6%, 0% inhibition, respectively (Fig. 8). Earlier, metal ions such as Mg^{2+} and Cu^{2+} were reported as activators at 1 mM by Kant et al. [12]. Kaur et al. [37] reported Mg^{2+} as inhibitor and Mn^{2+} and Fe^{2+} as activators of polygalacturonase.

Product identification

Thin Layer Chromatography results showed that purified enzyme digested the polygalacturonic acid into oligosaccharides with production of small amounts of galacturonic acid after long incubation time (Fig. 9). This result confirmed that the enzyme is an endo-polygalacturonase. Similar results were reported earlier by Li et al. [22] and Doostdar et al. [38].

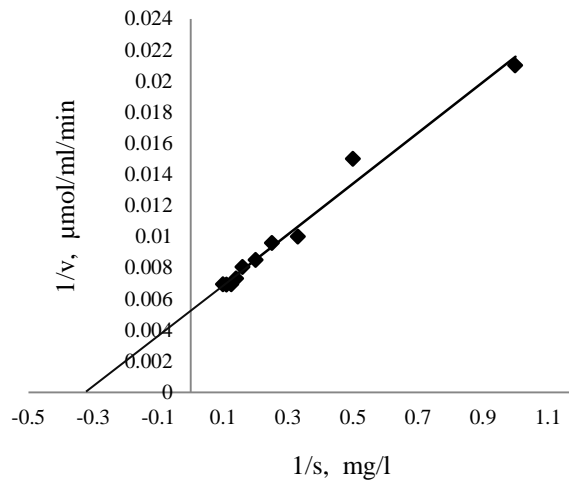


Fig. 7: Lineweaver-Burk plot for estimation of K_m and V_{max} of polygalacturonase enzyme from *A. niger* AN07.

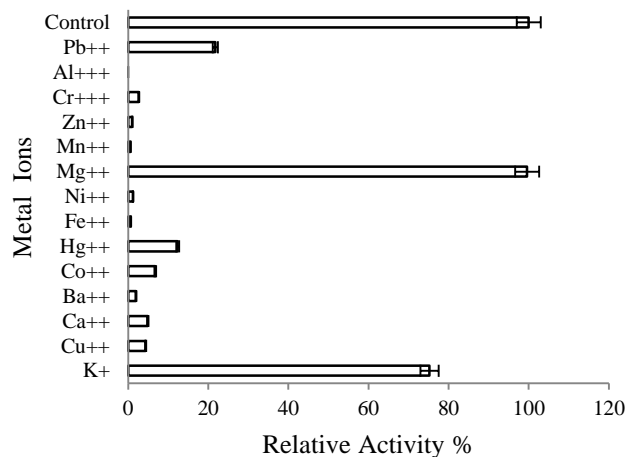


Fig. 8: Effect of metal ions on *A. niger* AN07 polygalacturonase activity.



Fig. 9: TLC using silica gel showing pattern of product formation by action of Endo-PGase on polygalacturonic acid at 55°C after different incubation time: Lane 1: control-1, only polygalacturonic acid; Lane 2: 0 min; Lane 3: 10 min; Lane 4: 20 min; Lane 5: 40 min; Lane 6: control-2, only galacturonic acid.

Conclusion

The polygalacturonase enzyme has been produced from *A. niger* AN07 using solid state fermentation. The enzyme has been purified by anion exchange and gel filtration chromatography to near homogeneity. The homogeneity of the purified enzyme and molecular weight was studied by Native and SDS-PAGE analysis, respectively. The purified enzyme is stable at pH ranging from 4-9 and temperature at 55°C for 1 h. Also, the thermo-tolerance, acid-alkali stability, and affinity towards the substrate, as shown by the low *K_m*, suggested that *A. niger* AN07 polygalacturonase can be an efficient and economic enzymatic option for food industries.

Acknowledgements

The authors acknowledge the facilities provided by the Department of Biotechnology, Ministry of Science and Technology, Government of India, New Delhi (DBT) in the School of Biotechnology under M.Sc. Biotechnology program and Distributed Bioinformatics sub center. The Authors also acknowledge the facilities of Department of Biosciences, Maharaja Ranjit Singh College of Professional Sciences, Indore.

Conflict of Interest

The authors declare that there is no conflict of interest in the present work.

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