Isolation and Characterization of polygalacturonase producing thermophilic Aspergillus spp. isolated from decayed tomato fruits

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Research Article

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Abstract

In this study, a polygalacturonase-producing fungus was isolated from decaying tomatoes. Based on colony morphology and hyphal characteristics, this fungus has been identified as *Aspergillus* sp. The fungus was used in solid-state fermentation to produce an acidic polygalacturonase enzyme. The crude extract obtained from solid-state fermentation had an activity of 94.6 U/mL. The enzyme was then purified using ammonium sulphate precipitation and column chromatography. Ammonium sulphate precipitation increased the enzyme's specific activity from 6.89 U/mg to 12.42 U/mg. Sephadex G-200 was used to purify the enzyme 3.58 times, and its specific activity was determined to be 24.66 U/mg. The Sephacryl S-100 column was responsible for achieving a final fold purification of 9.93 and a specific activity of 68.41 U/mg. When polygalacturonic acid was used as a substrate, the purified enzyme showed the best performance. The enzyme's optimum temperature and pH were found to be 55°C and 5, respectively. $CaCl_2$ was found to be the best chelating ion for the enzyme. This enzyme is recommended for use in a variety of industrial applications.

1. INTRODUCTION

Enzymes are now the cornerstone of several industries around the world, including pharmaceuticals, brewing, fabric, and most processed foods. As a result, the demand for enzyme time has risen dramatically (Cocok et al. 2017; Li et al. 2012; Ramesh et al. 2020; Raveendran et al. 2018). Pectinases are a class of pectinolytic enzymes that catalyse the depolymerisation and degradation of pectinaceous materials using hydrolases, lyases (depolymerisation reaction), or esterases (de-esterification reaction) (Zhang et al. 2021). Pectinase can also hydrolyze the alpha-1, 4 glycosidic linkages that exist between galacturonic acid residues and sugar (Rahman et al. 2019). Polygalacturonases, pectin lyase, and pectin methyl esterase are enzymes that hydrolyze the glycosidic bonds in pectic substances (Jayani et al. 2010; Khatri et al. 2015; Wang et al. 2015). Polygalacturonase is a depolymerizing enzyme that catalyses the − 1,4 glycosidic linkage in the pectin chain, resulting in galacturonic acid units (Ahmed et al. 2021). Polygalacturonase's biotechnological potential is expanding due to increasing applications in the food and feed industries. It belongs to the pectinase enzyme family, which accounts for 25% of all industrial enzymes worldwide (Munir et al. 2019). Polygalacturonases are the most exhaustively studied pectinolytic enzyme family (Jayani et al. 2005).

Microorganisms account for the majority of industrial demand for enzymes. Microorganisms are preferred in the industry for enzyme production due to their high growth capability, short life span, and ease of genetic manipulation (Haile & Ayele, 2022). Microbial pectinolytic enzymes, which are mostly produced by fungi, are used in a variety of large-scale industrial processes (Soares et al., 2012). Filamentous fungi are the primary source of hydrolases because they produce multienzyme complexes composed of endo- and exo-enzymes that degrade polymers such as cellulose, hemicellulose, and pectin (Ramos-Ibarra et al., 2017). Commercial pectinases are primarily derived from *Aspergillus* (Ravi et al., 2017). *Aspergillus* sp pectinases are widely used in industry because this strain has GRAS (Generally Regarded As Safe) status, which means that the metabolites produced by this strain can be used safely.

This fungus produces pectinases such as polymethyl-galacturonase (PMG), polygalacturonase (PG), and pectin esterase (PE) (Reddy and Sreeramulu, 2012). The addition of commercial pectinolytic enzyme preparations greatly improves pectinolytic enzyme preparation and increases juice yield (Ribeiro et al., 2010). Thermophilic fungi are eukaryotes that have an exceptional ability to grow at high temperatures of 50°-60°C and can survive in a wide range of extreme environments (Majumdar et al., 2018). Furthermore, the maximum activation temperature of fungi-produced polygalacturonase enzymes is between 35 and 60 ° C. (Anand et al., 2016; Thakur et al., 2010).

The most common methods for producing enzymes are submerged and solid-state fermentation, but solid-state fermentation is more productive than the others (Karimi et al., 2021). Because solid-state fermentation has several advantages, such as high productivity, extended product stability, and low production costs, it is a preferred technique (Yoon et al., 2014). Rice bran, sugar cane bagasse, orange bagasse, sugar beet pulp, wheat bran, and other food processing waste are all suitable substrates for pectinase production via solid-state fermentation (Alavi et al., 2020). The study's primary objective was to identify an efficient fungal strain capable of producing Polygalacturonase. To achieve the aforementioned objective, comparatively less explored fruit sources such as tomatoes were investigated.

2. MATERIALS AND METHODS

2.1. Sample collection

The ripened fruit (Tomato) was collected from the Sikella and Secha Arba Minch fruit markets. These ripened fruits were transferred to the research laboratory of Advanced Biotechnology and Microbial Biotechnology, College of Natural Sciences, Arba Minch University (AMU), Ethiopia in sterilized polythene bags for further processing. These fruits were allowed to decay in the laboratory (Fig. 1).

2.2. Isolation and Primary Screening

To prepare a stock solution, the fruit samples were immersed in sterilised distilled water. Each stock was serially diluted twice and aseptically poured into plates containing mineral salt agar media (0.2g $NaNO_3$, 0.05g KCl, 0.05g $MgSO_4$, 0.02g K_2HPO_4, 0.01g $FeSO_4$, 1g pectin, and 2g agar/100mL). Plates were incubated at 50°C for five to seven days. Following incubation, plates were poured with a potassium iodide-iodine solution (1.5g potassium iodide and 0.3g iodine/100mL) to examine the pectin lysis zones on plates for primary screening of fungal isolates with minor modification (Munir et al., 2019).

2.3. Secondary screening

Solid-state fermentation was used for secondary screening for polygalacturonase estimation. Bagasse from sugarcane was extracted and dried to make powder. This powdered extract was then used as a carbon source in a fermentation medium modified by Acuna-Arguelles et al (1995). Aseptically inoculating 250 ml of fermentation medium with spore inoculum (2 ml). The inoculated fermentation medium was incubated at 50°C for 4–5 days. Furthermore, the fermented media was extracted with 30

mL of distilled water. The flasks were vigorously shaken for 1 hour before being filtered through cheesecloth. The crude enzyme was extracted by adding 100 mL of citrate buffer to each flask (0.1 M, pH 5.0). The extract was centrifuged at 4°C for 15 min at 10,000 rpm, and the supernatant was sieved using Whatman No. 1 filter paper to remove all spores. The obtained supernatant (crude enzyme) was used to estimate polygalacturonase.

2.4. Identification of Polygalacturonase producing Isolate

The fungus with the highest Polygalacturonase hydrolysis value was macromorphologically characterised by observing colony characteristics such as colour, texture, and spore structure following (Shamly et al., 2006). Micromorphologically, the conventional lactophenol cotton blue technique was used (LPCB).

2.5. Enzyme Purification

The culture filtrate was centrifuged at 10,000 rpm for 20 minutes at room temperature. The salting-out procedure was carried out following the method adopted by (Siddiqui et al., 2012). To achieve 20% saturation, solid ammonium sulphate was added slowly to the crude enzyme preparation supernatant. Ammonium sulphate was added to an ice bath with continuous stirring and then stored overnight at 4°C. The precipitated protein was removed by centrifugation at 4°C for 30 minutes at 10,000 rpm. After that, ammonium sulphate was added to the supernatant to bring it up to 80% saturation. Again, centrifugation at 4°C for 30 min at 10,000 rpm was used to separate the precipitated protein, which was then dissolved in sodium acetate buffer (0.1 M; pH 5.0) The crude enzyme was then loaded onto a Sephadex G-200 (1 50 cm) column that had been pre-equilibrated with sodium acetate buffer (0.1 M; pH 5.0). At a flow rate of 24 mL/h, 3 mL volume fractions were collected. The eluted fractions were monitored at 280 nm for protein and enzyme activity assays. The fractions with the highest polygalacturonase activity were loaded onto pre-equilibrated Sephacryl S-100 columns (1.6 cm 60 cm) at a flow rate of 20 mL/h. 1.5 mL fractions were collected and examined for protein and polygalacturonase activity regularly (Siddiqui et al., 2012).

2.6. Enzyme assay

Polygalacturonase activity was determined by measuring the releasing sugar group from citrus pectin using a 3, 5-dinitrosalicylic acid (DNSA) reagent assay, according to Adedayo et al. (2021). In a test tube, 2 ml of crude enzyme and 2 ml of citrus pectin were mixed in phosphate buffer and incubated at 50 ^{0}C for 30 minutes. After incubation, the mixture was filtered, and 2 mL of DNSA reagent was added to 2 mL of the filtrate to stop the reaction, and the mixture was kept in a boiling water bath at 1000C for 10 minutes until the yellow colour developed. The tubes were then cooled with running water. A spectrophotometer was used to measure the optical density of the resulting coloured solution at 540 nm. The amount of enzyme that released 1 mol of galacturonic acid per minute was defined as one unit of pectinase activity (U).

2.7. Protein estimation

The protein concentration was determined by Lowry's method, as described by Lowry (1951) using bovine serum albumin (BSA) as a standard, and absorbance was read at 660 nm using UV-UV-vis Spectrophotometer.

2.8. Characterization of the enzyme

Substrate specificity

Purified pectinase was evaluated for substrate specificity against polygalacturonic acid, pectin, xylan, galactose, and cellulose at 0.1% (w/v). The substrates were incubated with the purified enzyme for 4 hours at an optimal temperature in 50 mM citrate buffer (pH 4.4). Polygalacturonase activity was determined for each substrate using standard methods, with pectin serving as a control (Thakur et al., 2010).

Effect of temperature

The enzyme activity was determined by incubating the reaction mixture (as described in the enzyme assay method) at different temperatures in the range from 30 to 60 ◦C. The optimum temperature for polygalacturonase activity was calculated by plotting enzyme activity against temperatures.

Effect of pH

The effect of reaction pH on polygalacturonase activity was accessed using citrate buffer (pH 2.0–4.0), and potassium-phosphate buffer (pH 5.0–8.0). The reaction mixture was incubated for 4 hr at 50°C.

Effect of divalent cations on enzyme activity

The effects of divalent cations on PG activity were determined by adding $CaCl_2$, $MgCl_2$, $MnSO_4$, and $FeCl_2$ at a 1 mM final concentration to the standard PG activity assay reaction. Reactions were conducted in duplicate and the experiment was repeated. The activity measured in the absence of cations was recorded as 100%.

3. RESULTS AND DISCUSSIONS

3.1. Isolation of fungi producing polygalacturonase

Twenty-five fungi strains were isolated from the cultures. Pure cultures were subcultured onto pectin agar media and kept for enzymatic studies and identification. Ten strains were isolated that were able to grow on a medium containing polygalacturonic acid as the sole carbon source. At pH 5.6, these strains were tested for polygalacturonic acid hydrolysis using a plate assay. Polygalacturonase activity was indicated by a clear zone against the media (Fig. 2). When presented with clear zones around colonies of at least 23 mm, the strains were classified as very good producers of pectin depolymerizing enzymes, good producers when the zones were at least 18 mm, weak producers when the zones were at least 15 mm,

and poor producers (Table 1) when no polygalacturonase activity and no clear zones were observed. The strain with the largest zone (approximately 30 mm) was used to produce enzymes in liquid media.

Table 1
Polygalacturonase activity of different isolated fungal strains

S/No	Polygalacturonase isolates	Zone of Inhibition in mm
1.	AT_1	20
2.	AT_3	23
3.	AT_5	19
4.	AT_7	17
5.	AT_{11}	21
6.	AT_{14}	25
7.	**AT_{16}**	**30**
8.	AT_{20}	20
9.	AT_{22}	15
10.	AT_{24}	18

3.2. Secondary screening

The secondary screening was performed on the selected pectinolytic fungal strains, AT3, 14, and 16. (Table 2). Among these, a fungal strain AT16 isolated from Arbaminch had the highest polygalacturonase production (25.67 ± 0.10 U/ml/min) and protein content (13.74 mg/ml), with production carried out using sugarcane bagasse dried powder as substrate.

Table 2
Secondary screening of polygalacturonase fungal isolates for polygalacturonase production

S/No	Polygalacturonase isolates	Polygalacturonase activity (U/ml/)
1	AT_3	19.83 ± 0.18
2	AT_{14}	16.57 ± 0.36
3	**AT_{16}**	**25.67 ± 0.10**

3.3. Identification of isolate

In Fig. 3, a fungus was isolated and identified based on cultural characteristics and sporulation as *Aspergillus* sp. Based on primary and secondary screening, *Aspergillus* sp. was identified as a potent strain and was chosen for further investigation. Initially, the colonies are made up of a compact yellow felt. The colonies then turn brown due to the production of conidiophores. The reverse side ranges from cream to yellow. And the hyphae had radiate conidial heads, and no teleomorph stage was observed, but there was heavy sporulation, the hyphae were septate and hyaline, and the conidia were globose to subglobose. *Aspergillus* sp. strains are promising sources of pectinase enzyme. Several previous studies have shown that *Aspergillus* sp. is important in the commercial production of polygalacturonase enzyme (Maciel et al., 2013, El Enshasy et al., 2018).

3.4. Production of Polygalacturonase

After solid-state fermentation, the crude enzyme was extracted. In 150 mL of crude extract, protein content and polygalacturonase enzyme activity were determined. The protein content was determined to be 13.74 mg/ml. The activity of polygalacturonase was determined to be 94.6 U/ml. The specific activity was found to be 6.89 U/mg. *Mucorgenevensis* has a maximum reported polygalacturonase activity of 5 U/mL (Alves et al., 2002), *Penicillium viridicatum* has an activity of 18 U/mL (Gomes et al., 2009), and *Mucorcircinelloides* has an activity of 9.15 U/mL (Thakur, 2010).

3.5. Enzyme Purification

PGase of the screened Aspergillus sp was purified from 500 mL of SSF crude extract (Table 3). Purification was carried out in two stages. First, ammonium sulphate precipitation was used to separate proteins with varying solubilities. The polygalacturonase enzyme was initially purified by adding solid ammonium sulphate. Ammonium sulphate precipitation increased the enzyme's specific activity from 6.89 U/mg to 12.42 U/mg. Polygalacturonase can be precipitated with 0–90% ammonium sulphate depending on the source of the enzyme (Mohammed et al., 2010; Shalom et al., 2017). Second, the sample was analyzed on two columns of chromatography, the first of which yielded 2.93 mg/ml of protein produced from 80 mL enzyme extract and purified 3.58 folds by Sephadex G -200, and the second of which yielded specific activity of 24.66 U/mL. The Sephacryl S-100 column aided in final fold purification, with yields of 9.93% and 20.97%, respectively. Previous researchers have found significant variations in yield and purification. Satapathy et al. (2021) found *Aspergillus parvisclerotigenus* to be 2.10-fold purified with a yield of 2.21%. *Penicillium oxalicum* was purified 29.9 times and yielded 17.1%, according to Cheng et al. (2016). *Penicillium oxalicum* was purified 28 times and yielded 57%, according to Almowallad et al. (2022). For *Aspergillus fumigatus*, Anand et al. (2016) reported 18.43-fold purification and 2.98% yield. These factors are primarily determined by the strain as well as the purification techniques used.

Table 3
Purification of polygalacturonase from *Aspergillus* sp

Purification steps	Collected Volume (mL)	Total Protein (mg/mL)	Total enzyme Activity (U/mL)	Specific Activity (U/mg)	Purification fold	Yield (%)
Crude extract	150	13.74	94.6	6.89	1	100
$(NH_4)_2 SO_4$ precipitation	80	2.93	36.4	12.42	1.8	38.48
SephadexG-200	12	1.16	28.6	24.66	3.58	30.23
Sephacryl S-100	1	0.29	19.84	68.41	9.93	20.97

3.6. Characterization of the enzyme

Effect of Temperature on the activity of polygalacturonase

Temperature is a critical factor in both microbial growth and product formation. The temperature of incubation has a significant impact on microbial growth rate, enzyme secretion, enzyme inhibition, and protein denaturation (Adeyefa and Ebuehi, 2020). The effect of reaction temperature on polygalacturonase activity is depicted in Fig. 4. The pectinase activity was detected at temperatures ranging from 30°C to 60°C, with 55°C being the optimal temperature, followed by 50°C and 45°C. The finding demonstrated that polygalacturonase activity increased with increasing temperature until the optimal temperature was reached. Meanwhile, polygalacturonase activity dropped dramatically above 60°C. The present study results are in line with those revealed by Kaur et al. (2004), who found that exo-polygalacturonase produced from thermophilic mould *Sporotrichum thermophile* was optimally active at 55°C. The present study's findings are in agreement with those of Kaur et al. (2004), who found that exo-polygalacturonase produced by the thermophilic mould *Sporotrichum thermophile* was most active at 55°C. Previous research has found that the optimal temperature for PGase activity produced by *Aspergillus awamori* NRC-F18 and *Rhizomucor pusillus* was 55oC. The decrease in enzyme activity at high temperatures is attributed to the denaturation of the enzymes (Almowallad et al., 2022).

Effect of Substrate specificity on the activity of polygalacturonase

Purified pectinase's affinity for substrates was determined, as shown in Fig. 5. When polygalacturonic acid was used as a substrate, the PGases specificity was highest. The enzyme has 100%, 51.02%, 16.07%, 8.47%, and 45.67% relative activity against polygalacturonic acid, pectin, xylan, galactose, and cellulose, respectively. Siddiqui et al. (2012) reported a similar observation in which polygalacturonic acid was used as a substrate in the substrate specificity study and 8.34 U/mL of polygalacturonase activity (100% relative activity) was obtained.

Effect of pH on the activity of polygalacturonase

The initial pH of the fermentation medium is critical in determining metabolite synthesis levels. The stability of the microbial metabolite is also affected by the medium's hydrogen ion concentration (Adeyefa and Ebuehi, 2020). pH is important in pectinase production because it promotes and regulates extracellular enzyme synthesis by microorganisms, particularly fungi (Siddiqui et al., 2012). Fungi, particularly Aspergillus species, have been shown to thrive in acidic or slightly alkaline environments (Yusuf, 2019). In Fig. 6, the present study found that pH 5 was the optimum pH for polygalacturonase activity (100% relative activity) produced by *Aspergillus* sp. The results agreed with those of Aminzadeh et al. (2020), who found that Polygalacturonase from *Tetracoccosporium* sp. was more active at acidic pH of 5. Polygalacturonase from *Aspergillus fumigatus* with 5 (Wang et al., 2015) with 5, *Penicillium oxalicum* CZ1028 with 5 (Cheng et al., 2016), *Thermoascus aurantiacus* (da Silva Martins et al., 2012) with 5, *Penicillium oxalicum* AUMC 4153 with 5 ((Almowallad et al., 2022), *Aspergillus tubingensis* with 5 (Tai et al., 2012).

Effect of divalent cations on enzyme activity

The effects of different metal ions were studied using a concentration of 1 mM of each metal ion in the reaction solution (Fig. 7). Only Ca^{+2} was found to increase PG activity among all metal ions, whereas Zn^{+2} inhibited enzyme activity. Metal ions may typically act as PGase's moral character endorsement to naturally inspire the process (Robinson, 2015). In the energetic location, the bending ability of the enzyme capacity typically has differential elasticity (Mohamad et al., 2015).

4. CONCLUSIONS

Polygalacturonases are an important member of the pectinase enzyme family with significant biotechnological and commercial potential. Polygalacturonase enzyme was purified from *Aspergillus* sp by ammonium sulphate precipitation and column chromatography, yielding 9.93-fold purification with a specific activity of 68.41 U/mg protein and 20.97% yield. The purified polygalacturonase was naturally acidic, with a pH optimum of 5.0. The optimal temperature for maximum enzyme activity was found to be 55^{o}C, indicating that the enzyme is resistant to heat. $CaCl_2$ was revealed to be the most effective chelating ion for the enzyme. Additional research is conducted to identify the fungus strain using molecular characterization. Furthermore, the homogeneity of the enzyme should be investigated using SDS PAGE. Polygalacturonase is typically produced by fungi and is essential for the production of organic vegetable oil, and fruit juice, and is used in animals as an easily degradable animal food.

Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose

Author Contributions

Jeyaramraja P R contributed to the study conception and design. Material preparation and data collection were performed by Gebiru Sinshaw. Both authors read and approved the final manuscript.

Data Availability declaration

Data will be made available on reasonable request.

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Figures

Figure 1

Decayed tomato fruits used in this study

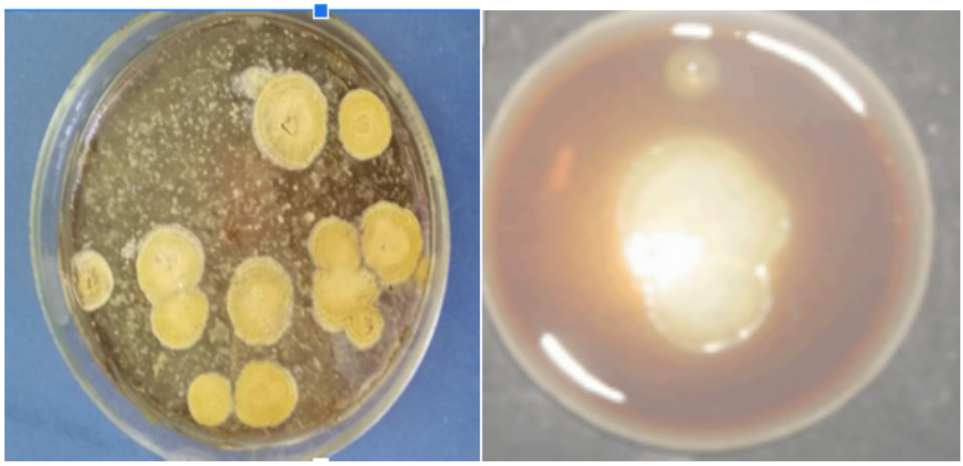

Figure 2

Fungal isolate culture, (b) Secondary screening for potential fungal isolate (Zone of polygalacturonic acid hydrolysis).

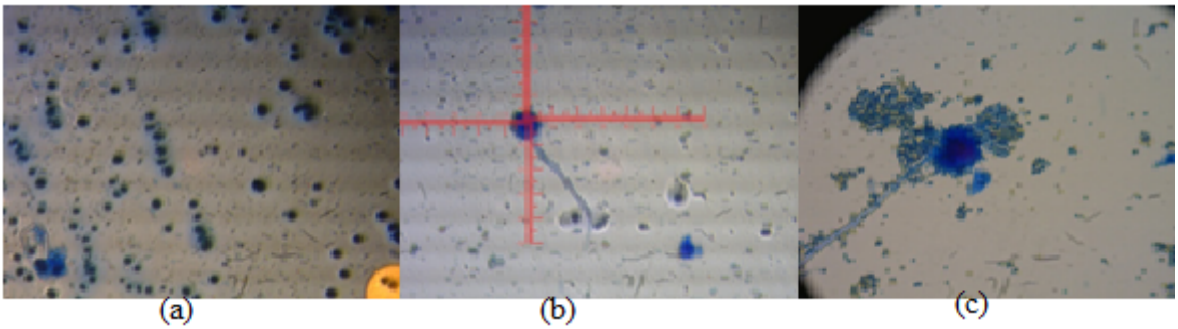

Figure 3

(a) Heavy sporulation of Isolate *Aspergillus* sp (b) Septate and hyaline hyphae of Isolate *Aspergillus* sp and (c) conidial head of Isolate *Aspergillus* sp.

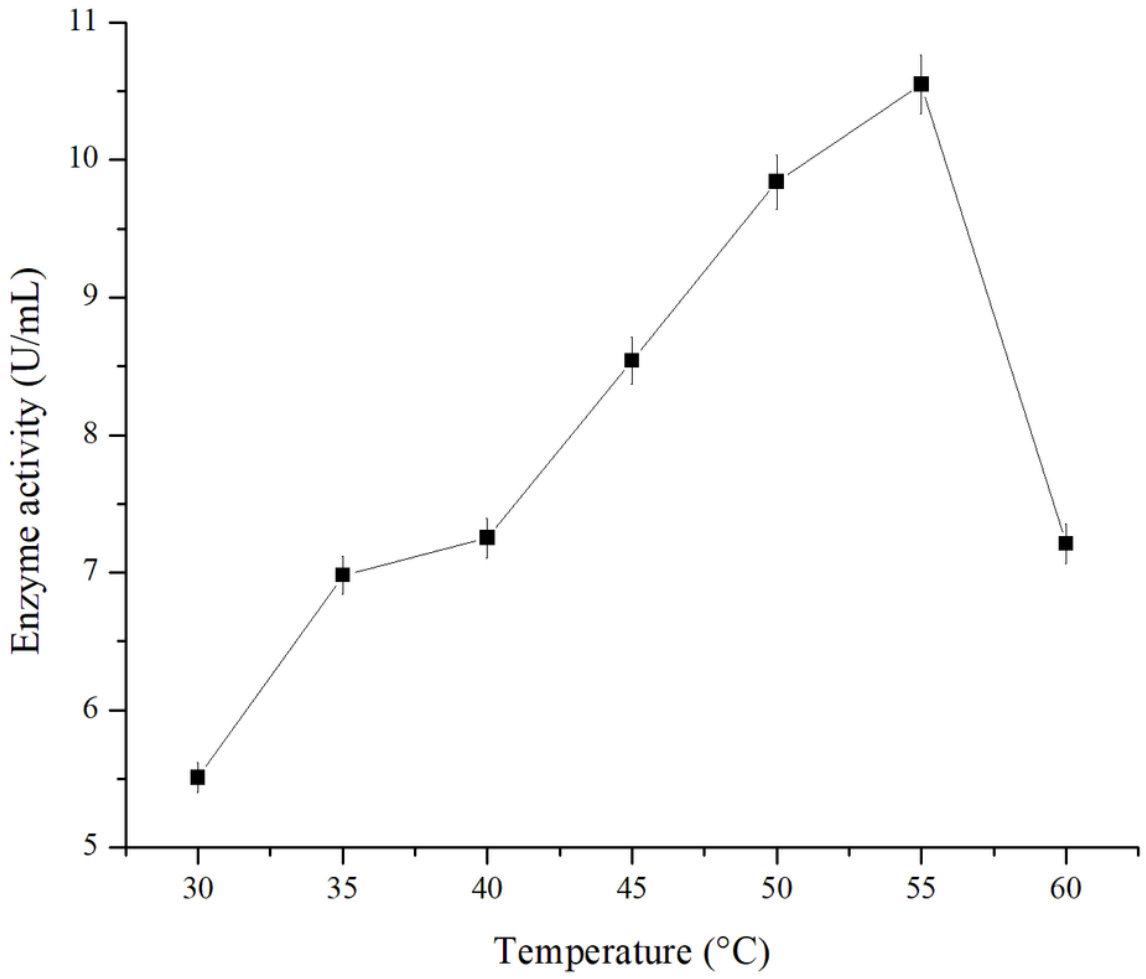

Figure 4

Effect of temperature on polygalacturonase activity from *Aspergillus* sp

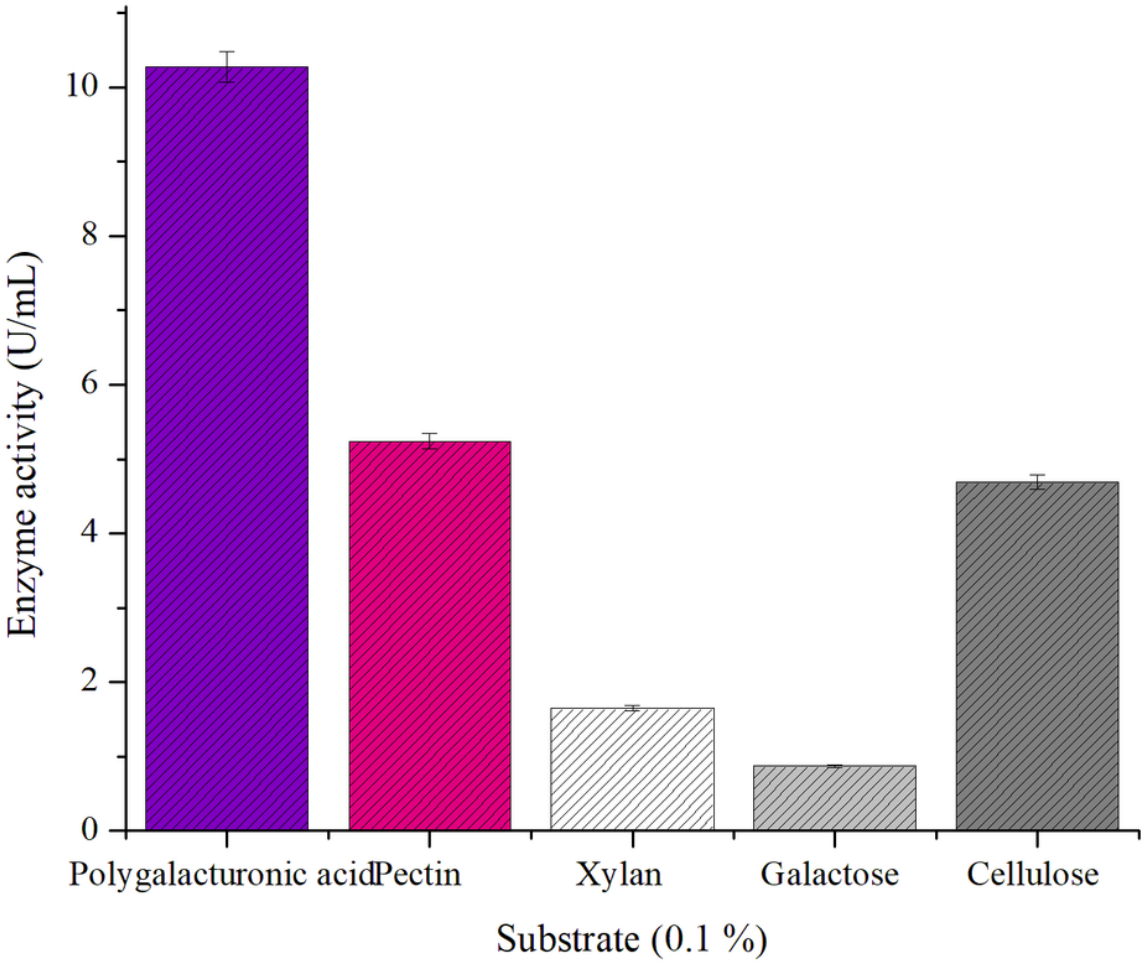

Figure 5

Substrate specificity of polygalacturonase from *Aspergillus* sp

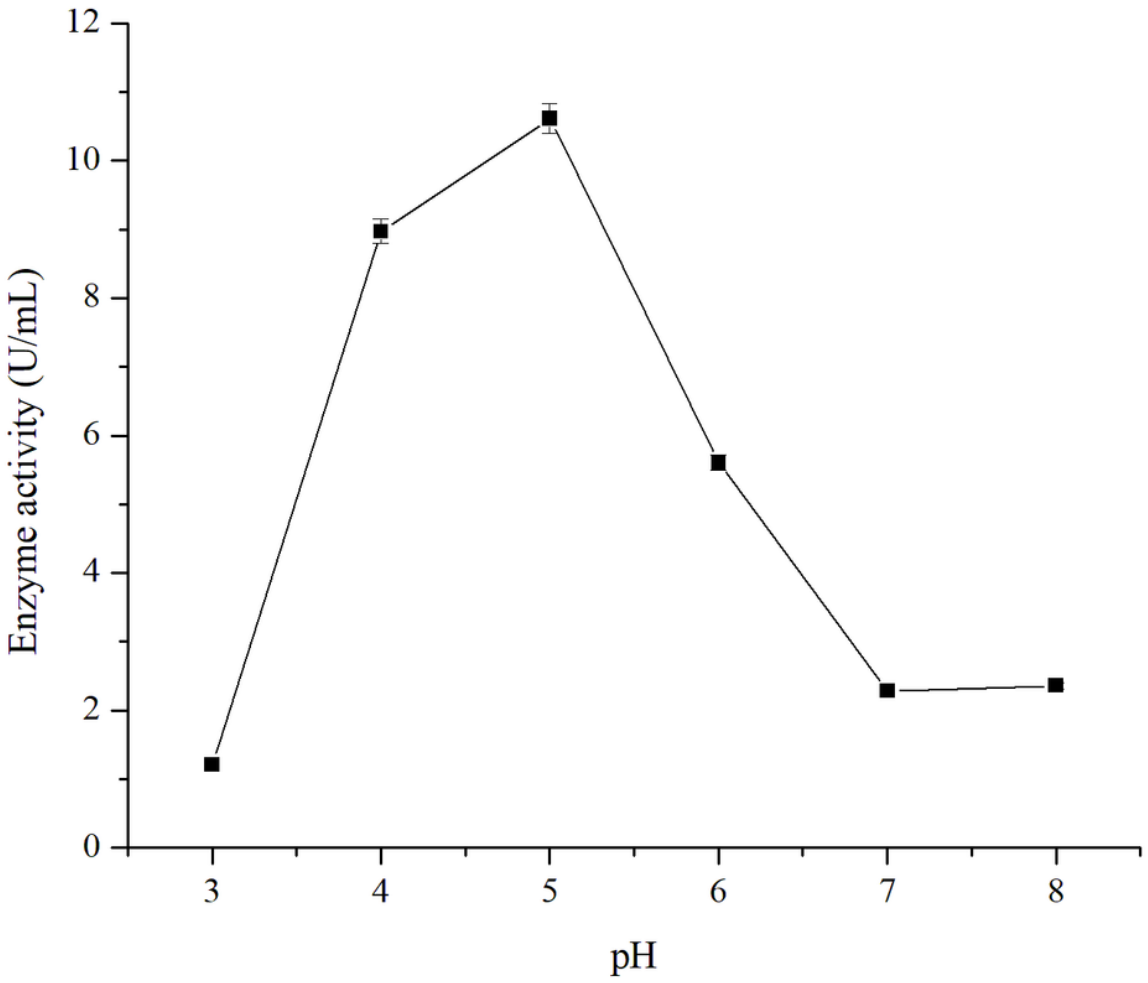

Figure 6

Effect of pH on polygalacturonase activity from *Aspergillus* sp

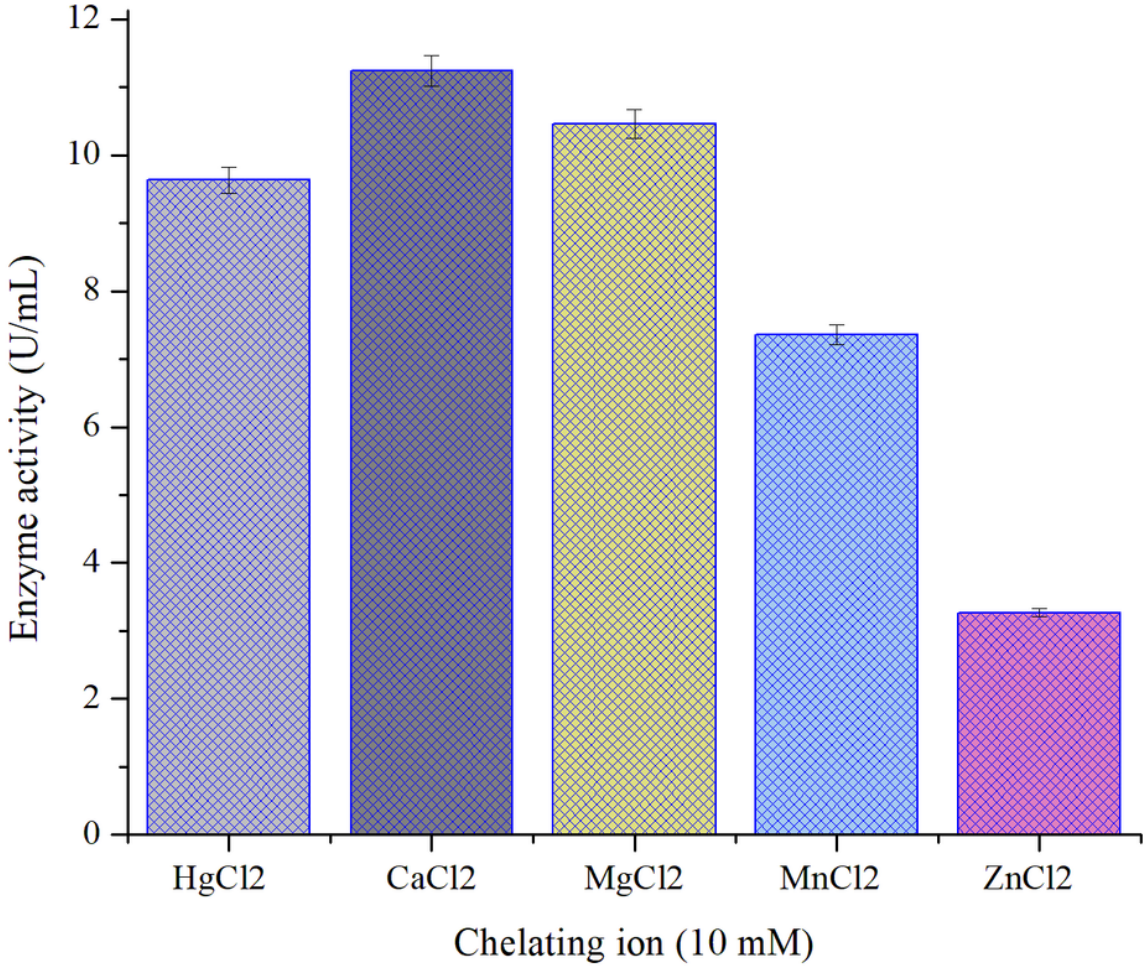

Figure 7

Effect of divalent cations on enzyme activity