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Isolation and characterization of polygalacturonase producing thermophilic *Aspergillus niger* isolated from decayed tomato fruits

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Column chromatography

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ABSTRACT

This study aimed to isolate a fungal strain capable of producing acidophilic and thermostable polygalacturonase. In this study, the fungal isolate was isolated from decaying tomatoes. Based on the colony characteristics, microscopic and morphological observations, the isolated fungal pathogen has been identified as *Aspergillus niger*. The isolated fungus was used in solid-state fermentation to produce an acidic polygalacturonase enzyme. The enzyme was then purified using ammonium sulphate precipitation and column chromatography, and its activity was assayed by measuring the releasing sugar group from citrus pectin using a 3, 5-dinitrosalicylic acid (DNSA) reagent assay. The crude extract obtained from solid-state fermentation had an activity of 94.6 U/mL. 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 achieved a final fold purification of 9.93 times and a specific activity of 68.41 U/mg. The purified enzyme performed best when polygalacturonic acid was used as a substrate. The enzyme's optimum temperature and pH were 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 as the enzyme was found to be stable at acidic pH and high temperature.

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1 Introduction

Enzymes are now the cornerstone of several industries worldwide, including pharmaceuticals, brewing, fabric, and most processed foods. As a result, the demand for enzymes has risen dramatically (Li et al. 2012; Cocok et al. 2017; Raveendran et al. 2018; Ramesh et al. 2020). 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 between galacturonic acid residues and sugar (Rahman et al. 2019). Polygalacturonases, pectin lyases, and pectin methyl esterases 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 α-1,4 glycosidic linkage in the pectin chain, resulting in galacturonic acid units (Ahmed et al. 2021). The biotechnological potential of polygalacturonase 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 and Ayele 2022). Microbial pectinolytic enzymes, produced mainly by fungi, are used in various large-scale industrial processes (Soares et al. 2012). Filamentous fungi are the primary sources 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 and Raghu 2017). *Aspergillus* sp. *pectinases* are widely used in industry because this strain has GRAS (Generally Recognized As Safe) status, which means that the metabolites produced by this strain can be used safely. This fungus produces pectinases such as polymethyl-galacturonase, polygalacturonase, and pectin esterase (Reddy and Sreeramulu 2012). Adding commercial pectinolytic enzyme preparations greatly improves the 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 (Thakur et al. 2010; Anand et al. 2016).

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). Solid-state fermentation has several advantages, such as high productivity, extended product stability, and low production costs (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 primary objective of this study was to identify an efficient fungal strain capable of producing polygalacturonase. To achieve this objective, comparatively less explored fruit sources such as tomatoes were investigated. Various endophytic and pathogenic fungi were reported to be present in tomato fruits (Paolo et al. 2018). Some endophytic fungi (Kaur et al. 2004) and pathogenic fungi (Samal et al. 2023) have been reported to produce a variety of industrially important enzymes. The diversity and interactions of endophytic and pathogenic fungi within tomato fruits present a fascinating area of research with the potential to uncover novel strategies for producing industrially important enzymes (Ikram et al. 2020). This study aimed to isolate and characterize the polygalacturonase-isolated enzyme from the thermophilic *Aspergillus niger* fungal pathogen found in decayed tomato fruits.

2 Materials And Methods

2.1 Sample collection

The ripened fruits of tomatoes were collected from a local fruit market in Ethiopia. These ripened fruits were transferred to the laboratory in sterilized polythene bags for further processing. These fruits were allowed to decay in the laboratory (Figure 1).

Figure 1 Decayed tomato fruits used in this study

2.2 Isolation of fungi and primary screening

The fruit samples were immersed in sterilised distilled water to prepare a stock solution. 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 modifications (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 prepared according to Acuña-Argüelles et al. (1995). The fermentation medium (250 ml) was inoculated aseptically 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 activity as per the method of Adedayo et al. (2021).

2.4 Identification of polygalacturonase-producing isolate

The fungus with the highest polygalacturonic acid hydrolysis value was identified by observing hyphal characteristics colony characteristics such as colour, texture, and spore structure following the methodology of Shamly et al. (2014). The conventional lactophenol cotton blue technique (LPCB) was used to study the fungal morphology.

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 of Siddiqui et al. (2012). To achieve 20% saturation, solid ammonium sulphate was added slowly to the crude enzyme preparation in 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 reach 80% saturation. Again, centrifugation at 4°C for 30 min at 10,000 rpm separated 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 (150 cm) column 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 using a spectrophotometer at 280 nm for protein and enzyme activity. The fractions with the highest polygalacturonase activity were loaded onto pre-equilibrated Sephacryl S-100 columns (1.6 cm X 60 cm) at a flow rate of 20 ml/h. Fractions (1.5 ml) were collected and examined regularly for protein and polygalacturonase activity.

2.6 Enzyme assay

Polygalacturonase activity was determined by measuring the releasing sugar group from citrus pectin using 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°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 100°C for 10 minutes until 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 as described by Lowry et al. (1951) using bovine serum albumin (BSA) as standard, and absorbance was read at 660 nm using a UV-Vis spectrophotometer.

2.8 Characterization of the enzyme

2.8.1 Substrate specificity

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

2.8.2 Effect of temperature

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

2.8.3 Effect of pH

The effect of reaction pH on polygalacturonase activity was assessed using citrate buffer (pH 2.0–4.0) and potassium-phosphate buffer (pH 5.0–8.0) following the method of Bentouhami et al. (2024). The reaction mixture was incubated for 4 hr at 50°C. Finally, the enzyme activity was determined to evaluate its stability in varying ionic strengths.

2.8.4 Effect of divalent cations on enzyme activity

The divalent cations tested include $CaCl_2$, $MgCl_2$, $MnSO_4$, and $FeCl_2$. To study the effect of various divalent cations on enzyme activity, 2 ml of crude enzyme and 2 ml of citrus pectin were mixed in phosphate buffer containing divalent cations to a final concentration of 10 mM and incubated at 50°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. The remaining steps were performed as given in section 2.6.

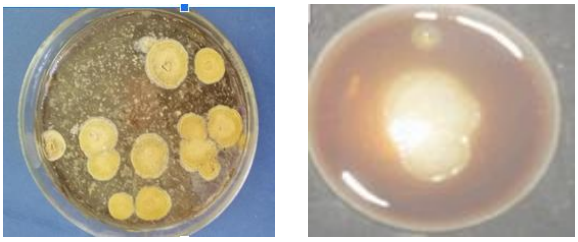
2.9 Statistical analysis

The experiments were conducted following a completely randomized block design. Each experiment was repeated three times to get triplicate data subjected to statistical analysis using the SPSS tool to compute mean and standard error (SE) values.

3 Results and Discussion

3.1 Isolation of fungi producing polygalacturonase

Fungi secrete various enzymes that can degrade complex plant-derived polysaccharides, such as cellulose and pectin (Badhan et al. 2018). Among the pectinolytic enzymes, polygalacturonase plays a crucial role in the degradation of polygalacturonic acid, the main component of pectin (Pedrolli et al. 2009). The activity of polygalacturonase can lead to the formation of clear zones in nutrient media, a widely used phenotypic characteristic for identifying and screening fungal isolates (Balabanova et al. 2018). The present study isolated twenty-five fungal strains from infected tomato fruits. Pure cultures of isolated fungal strains were sub-cultured onto pectin agar media and kept for enzymatic studies and identification. Among these twenty-five pure cultured fungal strains, ten strains that were able to grow on a medium containing polygalacturonic acid as the sole carbon source were isolated. At pH 5.6, these ten strains were tested for polygalacturonic acid hydrolysis using a plate assay. Polygalacturonase activity of a strain was indicated by a clear zone in the media (Figure 2). When a fungal strain presented at least 23 mm clear zones around colonies, the strains were classified as very good producers of pectin depolymerizing enzymes, while if the zones were at least of



(a) **(b)**

Figure 2 (a) Fungal isolate culture, (b) Secondary screening for potential fungal isolate (Zone of polygalacturonic acid hydrolysis).

Table 1 Polygalacturonase activity of different isolated fungal strains

S.N.	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

18 mm and 15 mm these strains were considered as good producers and weak producers, respectively. Finally, the strain was considered poor producers when no polygalacturonase activity and clear zones were observed (Table 1). The strain with the most extensive zone (approximately 30 mm) was used for further parameter evaluation and to produce enzymes in liquid media.

3.2 Secondary screening

Solid-state fermentation has emerged as a promising approach for producing various enzymes, including polygalacturonase, a key enzyme involved in pectin degradation (Madamwar et al. 1989). Sugarcane bagasse, a readily available agricultural waste, has been extensively investigated as a carbon-rich substrate for solid-state fermentation due to its lignocellulosic composition (Garcia et al. 2018; Lamounier et al. 2018). The present study aims to explore the potential of utilizing sugarcane bagasse as a renewable and cost-effective carbon source for producing polygalacturonase by fungi in a solid-state fermentation system. 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 the tomato samples collected from Arba Minch 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.N.	Polygalacturonase isolates	Polygalacturonase activity (U/ml/Min)
1	AT_3	19.83 ± 0.18
2	AT_{14}	16.57 ± 0.36
3	AT_{16}	25.67 ± 0.10

Values are mean of triplicates followed by ±SE

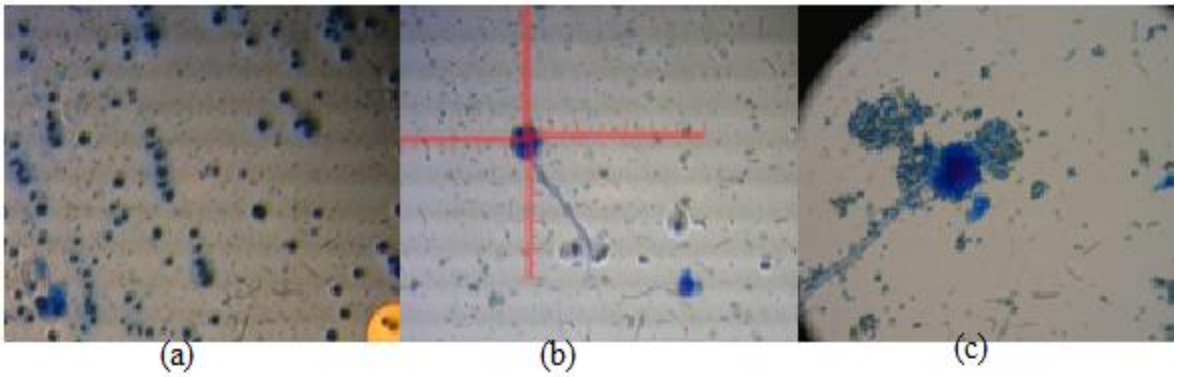


Figure 3 (a) Heavy sporulation of Isolate *Aspergillus niger* (b) Septate and hyaline hyphae of Isolate *Aspergillus niger* and (c) conidial head of Isolate *Aspergillus niger*

3.3 Identification of isolate

Isolated fungus strain was identified as *Aspergillus niger* based on morphological observation, cultural, hyphal characteristics and sporulation (Figure 3). This fungus was chosen for further investigation. Initially, the colonies were made up of a compact yellow felt, which later turned brown due to the conidiophores production, which is a characteristic of *A. niger* (Raper and Fennell 1965). The reverse side of the plate ranges from cream to yellow, which aligns with descriptions in the literature (Samson et al. 2010). The hyphal characteristics, including septate and hyaline hyphae, along with the conidial heads that radiate with heavy sporulation, are key identifying features of *A. niger* (Klich 2002). The absence of a teleomorph stage further supports the identification, as *A. niger* is typically known for its asexual reproduction (Gams et al. 1986). The conidia being globose to sub-globose is another hallmark trait corroborating the identification (Pitt and Hocking 2009).

3.4 Production of polygalacturonase

In the crude extract (150 ml) obtained using solid-state fermentation involving AT3, AT14, and AT16 strains, the protein content and polygalacturonase enzyme activity were found to be 13.74 mg/ml and 94.6 U/ml, respectively. Hence, the specific activity was 6.89 U/mg. The polygalacturonase activity recorded in the present study was higher than that reported by Alves et al. (2002) in the case of *Mucor genevensis* (5 U/ml) and Thakur et al. (2010) in *M. circinelloides* (9.15 U/ml), while the present study polygalacturonase activity was lower than the value reported by Gomes et al. (2009) in case of Penicillium viridicatum where the activity recorded, was 18 U/ml.

3.5 Enzyme purification

Polygalacturonase from the screened *A. niger* was purified from 150 mL of crude extract obtained from solid-state fermentation (Table 3). The polygalacturonase enzyme was initially purified by the salting out procedure, which involves the addition of up to 80% 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 (Buga et al. 2010; Chinedu et al. 2016). Sephadex G-200 column chromatography helped purify the enzyme 3.58 times, resulting in a specific activity of 24.66 U/mg. Finally, Sephacryl S-100 column chromatography increased the specific activity to 68.41 U/mg, which, compared to that of crude extract, was 9.93 times higher. The yield of the enzyme decreased as the purification fold increased.

Previous researchers have found significant variations in the purification fold and yield of polygalacturonase enzymes from various microbial sources. Cheng et al. (2016) isolated an acid-stable endo-polygalacturonase from *P. oxalicum* CZ1028. The purification process involved ammonium sulfate precipitation, hydrophobic interaction chromatography, anion exchange chromatography, and size exclusion chromatography. This resulted

Table 3 Purification of polygalacturonase from *A. niger*

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

in the enzyme purifying 29.9 times, achieving a specific activity of 2320 U/mg, with a final yield of 17.1%. Anand et al. (2016) purified polygalacturonase from *A. fumigatus* MTCC 2584 using acetone precipitation and Sephadex G-100 column, which resulted in 18.43 fold increase in enzyme purity, with a specific activity of 38.9 U/mg. Satapathy et al. (2021) extracted pectinase from *A. parvisclerotigenus* KX928754 using apple pomace as the substrate. The crude filtrate underwent ammonium sulfate precipitation, dialysis, and elution on a Sephadex G-100 column. As a result, the enzyme was purified 2.10-fold, with a yield rate of 2.91% and a specific activity of 1081.66 U/mg. Almowallad et al. (2022) partially purified exo-polygalacturonase from *P. oxalicum* AUMC 4153 using sugar beet manufacturing waste as the sole carbon source. The enzyme purification process included ammonium sulfate precipitation, acetone precipitation, and gel filtration chromatography. This resulted in a 28-fold increase in enzyme purity, with a final yield of 57%.

3.6 Characterization of the enzyme

3.6.1 Effect of temperature on the activity of polygalacturonase

Temperature is a critical factor in both microbial growth and product formation. The incubation temperature significantly impacts 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 Figure 4. Enzyme activity was detected at temperatures ranging from 30 to 60°C, with 55°C being the optimal temperature, followed by 50°C and 45°C. This finding demonstrated that polygalacturonase activity increased with increasing temperature until the optimal temperature was reached. However, polygalacturonase activity dropped dramatically above 55°C. The present study results align with those that Kaur et al. (2004) revealed, who found that exo-polygalacturonase produced from thermophilic mould *Sporotrichum thermophile* was optimally active at 55 °C. The decrease in enzyme activity at very high temperatures is attributed to the denaturation of the enzymes (Almowallad et al. 2022).

3.6.2 Effect of various substrates on the activity of polygalacturonase

The purified enzyme's affinity for different substrates was determined (Figure 5). The best substrates were polygalacturonic acid, pectin, cellulose, xylan, and galactose. Siddiqui et al. (2012) observed a maximal enzyme activity of 8.34 U/ml when polygalacturonic acid was used as a substrate. In the present study, the substrate polygalacturonic acid led to a maximum enzyme activity of 10.1 U/ml.

3.6.3 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 polygalacturonase 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 (Ahmed Olaitan 2019). The present

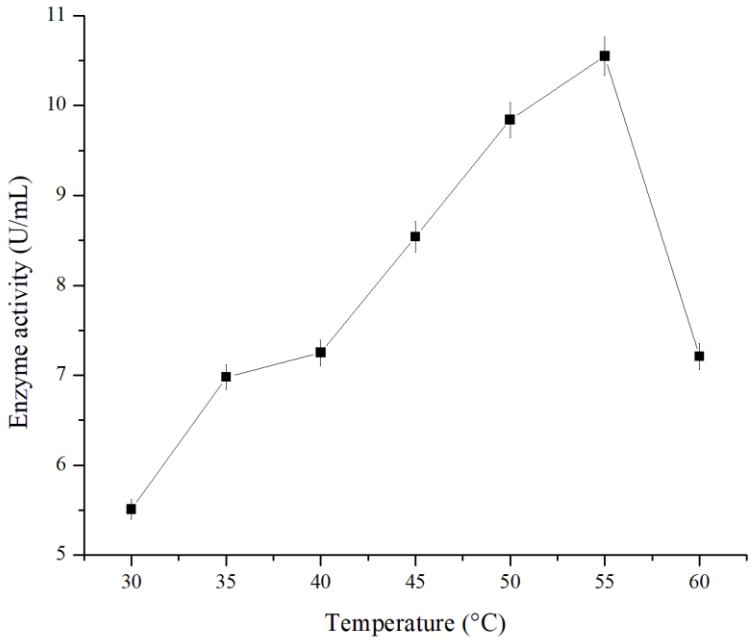


Figure 4 Effect of temperature on polygalacturonase activity from *Aspergillus niger*; values are mean of triplicates; error bar indicates ±SE.

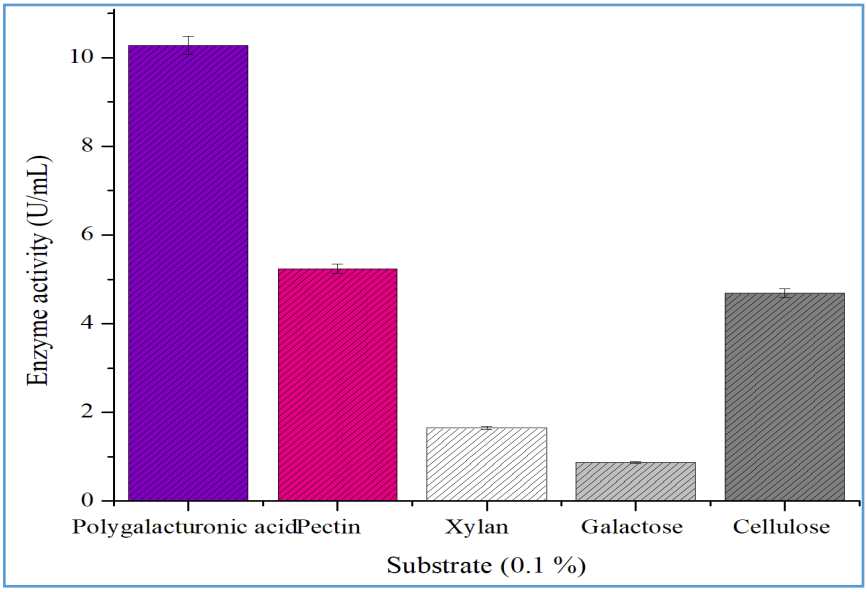


Figure 5 Substrate specificity of polygalacturonase from *Aspergillus niger*; values are mean of triplicates; error bar indicates ±SE.

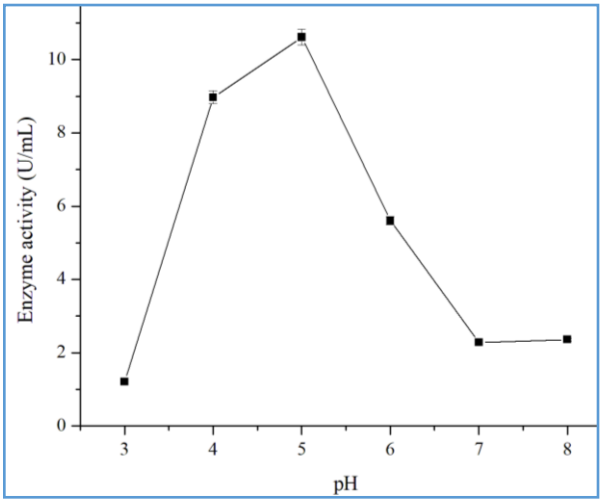


Figure 6 Effect of pH on polygalacturonase activity from *Aspergillus niger*; values are mean of triplicates; error bar indicates ±SE.

study found that pH 5 was the optimum for polygalacturonase activity (100% relative activity) produced by *A. niger* (Figure 6). The results agreed with Aminzadeh et al. (2007), who found that polygalacturonase from *Tetracoccosporium* sp. was more active at an acidic pH of 5. Similar observations were reported about the optimum pH for polygalacturonase from *A. fumigatus* (Wang et al. 2015), *P. oxalicum* CZ102 (Cheng et al. 2016), *Thermoascus aurantiacus* (Martins et al. 2012), *P. oxalicum* AUMC 4153 (Almowallad et al. 2022) and *A. tubingensis* (Tai et al. 2013).

3.6.4 Effect of divalent cations on enzyme activity

The effects of different metal ions on enzymatic activity were studied using a concentration of 10 mM of each metal ion in the reaction solution (Figure 7). Among all the divalent cations, Ca^{2+} was the optimum for the maximal polygalacturonase activity. On the contrary, Zn^{2+} inhibited the enzyme activity. The mechanism behind the increased activity of fungal polygalacturonases in the presence of certain divalent cations is not fully understood, but it is believed to involve several factors. First, divalent cations may help stabilize the enzyme structure, increasing catalytic efficiency (Pedrolli et al. 2009). Additionally, these cations may facilitate the interaction between the enzyme and the pectin substrate, a complex polysaccharide composed of galacturonic acid residues (Balabanova et al. 2018). Furthermore, divalent cations can influence the charge and conformation of the pectin substrate, making it more accessible to the polygalacturonase enzyme (Oumer 2017).

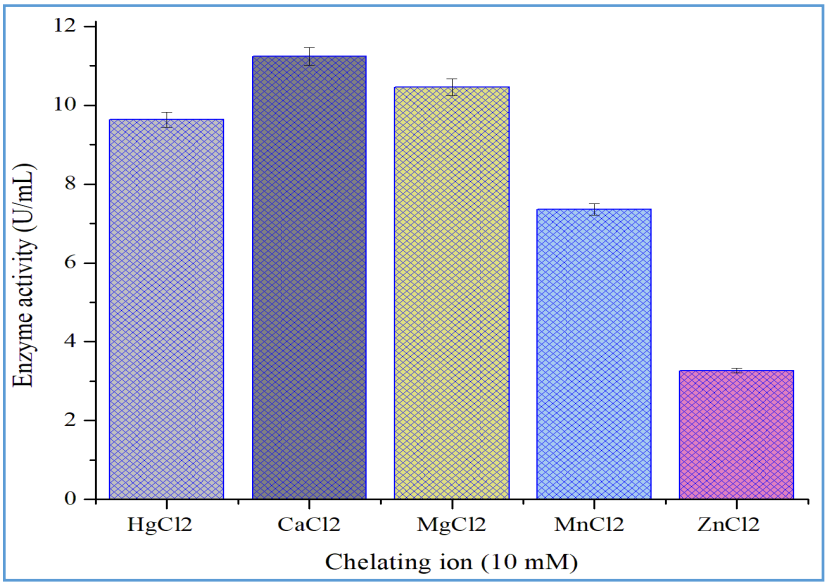


Figure 7 Effect of divalent cations on enzyme activity; values are mean of triplicates; error bar indicates ±SE.

Conclusion

Polygalacturonases are important members of the pectinase enzyme family and have significant biotechnological and commercial potential. In the current study, polygalacturonase was purified from *A. niger* 9.93 times by ammonium sulphate precipitation and column chromatography, resulting in specific activity of 68.41 U/mg protein. The purified polygalacturonase was naturally acidic, with an optimum pH of 5.0. The optimal temperature for maximum enzyme activity was 55°C, indicating that the enzyme is resistant to heat. $CaCl_2$ was revealed to be the most effective chelating ion for the enzyme. The homogeneity of the enzyme will be investigated using SDS PAGE shortly. Fungi typically produce polygalacturonase, which is essential for producing organic vegetable oil and fruit juice and being used to manufacture easily digestible animal feed.

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Competing interests

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

Data statement

Data will be made available on reasonable request.

Authors' contribution

Gebiru S conceived the idea and performed the isolation of fungi, solid state fermentation, and enzyme purification. Jeyaramraja P R supervised the entire work. Sasikumar J M contributed in manuscript drafting. Abate Ayele performed enzyme assays. All authors have read and approved the final manuscript for publication.

References

Acuña-Argüelles, M. E., Gutiérrez-Rojas, M., Viniegra-González, G., & Favela-Torres, E. (1995). Production and properties of three pectinolytic activities produced by *Aspergillus niger* in submerged and solid-state fermentation. *Applied Microbiology and Biotechnology*, *43*(5), 808–814. https://doi.org/10.1007/BF02431912

Adedayo, M. R., Mohammed, M. T., Ajiboye, A. E., & Abdulmumini, S. A. (2021). Pectinolytic activity of *Aspergillus niger* and *Aspergillus flavus* grown on grapefruit (citrus Parasidis) peel in solid state fermentation. *Global Journal of Pure and Applied Sciences*, *27*(2), 93–105. https://doi.org/10.4314/gjpas.v27i2.2

Adeyefa, O., & Ebuehi, O. (2020). Isolation, Identification and Characterization of Pectinase Producers from Agro Wastes (*Citrus sinensis* and *Ananas comosus*). *World Journal of Agriculture and Soil Science*, *4*(3). https://doi.org/10.33552/WJASS.2020.04.000589

Ahmed, J., Thakur, A., & Goyal, A. (2021). Emerging trends on the role of recombinant pectinolytic enzymes in industries- an overview. *Biocatalysis and Agricultural Biotechnology*, *38*, 102200. https://doi.org/10.1016/j.bcab.2021.102200

Ahmed Olaitan, Y. (2019). *Characterization and Partial Purification of Pectinase Produced by Aspergillus niger Using Banana Peel as Carbon Source* [Kwara State University]. Retrieved from https://www.proquest.com/openview/474b6ae1c0692a0a4f9d6ecdae26e885/1?pq-origsite=gscholar&cbl=18750&diss=y

Alavi, A., Nia, F. T., & Shariati, F. P. (2020). Polygalacturonase Production by *Aspergillus niger* Solid State Fermentation on Barley Bran and Sugar Beet Pulp Mixture. *Advanced Journal of Chemistry-Section A*, *3*(3), 350–357. https://doi.org/10.33945/SAMI/AJCA.2020.3.13

Almowallad, S. A., Alshammari, G. M., Alsayadi, M. M., Aljafer, N., Al-Sanea, E. A., Yahya, M. A., & Al-Harbi, L. N. (2022). Partial Purification and Characterization of Exo-Polygalacturonase Produced by Penicillium oxalicum AUMC 4153. *Life*, *12*(2), 284. https://doi.org/10.3390/life12020284

Alves, M. H., Campos-Takaki, G. M., Porto, A. L. F., & Milanez, A. I. (2002). Screening of *Mucor* spp. For the production of amylase, lipase, polygalacturonase and protease. *Brazilian Journal of Microbiology*, *33*(4). https://doi.org/10.1590/S1517-83822002000400009

Aminzadeh, S., Naderimanesh, H., Khajeh, K., & Sooudi, M. (2007). Isolation and Characterization of Polygalacturonase produced by *Tetracoccosporium* Sp. *Iranian Journal of Chemistry and Chemical Engineering*, *26*(1), 47–54.

Anand, G., Yadav, S., & Yadav, D. (2016). Purification and characterization of polygalacturonase from *Aspergillus fumigatus* MTCC 2584 and elucidating its application in retting of *Crotalaria juncea* fiber. *3 Biotech*, *6*(2), 201. https://doi.org/10.1007/s13205-016-0517-4

Badhan, A., Huang, J., Wang, Y., Abbott, D. W., Di Falco, M., Tsang, A., & McAllister, T. (2018). Saccharification efficiencies of multienzyme complexes produced by aerobic fungi. *New Biotechnology*, *46*, 1–6. https://doi.org/10.1016/j.nbt.2018.05.003

Balabanova, L., Slepchenko, L., Son, O., & Tekutyeva, L. (2018). Biotechnology Potential of Marine Fungi Degrading Plant and Algae Polymeric Substrates. *Frontiers in Microbiology*, *9*, 1527. https://doi.org/10.3389/fmicb.2018.01527

Bentouhami, N. E., Asehraou, A., Mechri, S., Hasnaoui, I., Moumnassi, S., et al. (2024). Purification and biochemical characterization of a novel thermostable endo-polygalacturonase from *Aspergillus niger* strain HO32 and its suitability for clarification of orange juice. *Process Biochemistry*, *145*, 63–73. https://doi.org/10.1016/j.procbio.2024.06.013

Buga, M. L., Ibrahim, S., & Nok, A. J. (2010). Partially purified polygalacturonase from *Aspergillus niger* (SA6). *African Journal of Biotechnology*, *9*(52), 8944–8954.

Cheng, Z., Chen, D., Lu, B., Wei, Y., Xian, L., Li, Y., Luo, Z., & Huang, R. (2016). A Novel Acid-Stable Endo-Polygalacturonase from *Penicillium oxalicum* CZ1028: Purification, Characterization, and Application in the Beverage Industry. *Journal of Microbiology and Biotechnology*, *26*(6), 989–998. https://doi.org/10.4014/jmb.1511.11045

Chinedu, S. N., Dayo-Oduko, O. P., & Iheagwam, F. N. (2016). Partial Purification and Kinetic Properties of Polygalacturonase from *Solanum macrocarpum* L. Fruit. *Biotechnology(Faisalabad)*, *16*(1), 27–33. https://doi.org/10.3923/biotech.2017.27.33

Cocok, A. M. B., Fahrurrozi, F., & Anja, M. (2017). Pectinase Production and Clarification Treatments of Apple (*Malus Domestica*) Juice. *Annales Bogorienses*, *21*(2), 63–68.

Gams, W., Christensen, M., Onions, A. H., Pitt, J. I., & Samson, R. A. (1986). Infrageneric Taxa of *Aspergillus*. In R. A. Samson & J. I. Pitt (Eds.), *Advances in Penicillium and Aspergillus Systematics* (pp. 55–62). Springer US. https://doi.org/10.1007/978-1-4757-1856-0_5

Garcia, N. F. L., Santos, F. R. D. S., Bocchini, D. A., Paz, M. F. D., Fonseca, G. G., & Leite, R. S. R. (2018). Catalytic properties of cellulases and hemicellulases produced by *Lichtheimia ramosa*: Potential for sugarcane bagasse saccharification. *Industrial Crops and Products*, *122*, 49–56. https://doi.org/10.1016/j.indcrop.2018.05.049

Gomes, E., Leite, R. S. R., Da Silva, R., & Silva, D. (2009). Purification of an Exopolygalacturonase from *Penicillium viridicatum RFC3* Produced in Submerged Fermentation. *International Journal of Microbiology*, *2009*, 1–8. https://doi.org/10.1155/2009/631942

Haile, S., & Ayele, A. (2022). Pectinase from Microorganisms and Its Industrial Applications. *The Scientific World Journal*, *2022*, 1–15. https://doi.org/10.1155/2022/1881305

Ikram, M., Ali, N., Jan, G., Jan, F. G., & Khan, N. (2020). Endophytic Fungal Diversity and their Interaction with Plants for Agriculture Sustainability Under Stressful Condition. *Recent Patents on Food, Nutrition & Agriculture*, *11*(2), 115–123. https://doi.org/10.2174/2212798410666190612130139

Jayani, R. S., Saxena, S., & Gupta, R. (2005). Microbial pectinolytic enzymes: A review. *Process Biochemistry*, *40*(9), 2931–2944. https://doi.org/10.1016/j.procbio.2005.03.026

Jayani, R. S., Shukla, S. K., & Gupta, R. (2010). Screening of Bacterial Strains for Polygalacturonase Activity: Its Production by *Bacillus sphaericus* (MTCC 7542). *Enzyme Research*, *2010*, 1–5. https://doi.org/10.4061/2010/306785

Karimi, F., Mazaheri, D., Saei Moghaddam, M., Mataei Moghaddam, A., Sanati, A. L., & Orooji, Y. (2021). Solid-state

fermentation as an alternative technology for cost-effective production of bioethanol as useful renewable energy: A review. *Biomass Conversion and Biorefinery*. https://doi.org/10.1007/s13399-021-01875-2

Kaur, G., Kumar, S., & Satyanarayana, T. (2004). Production, characterization and application of a thermostable polygalacturonase of a thermophilic mould *Sporotrichum thermophile* Apinis. *Bioresource Technology*, *94*(3), 239–243. https://doi.org/10.1016/j.biortech.2003.05.003

Khatri, B. P., Bhattarai, T., Shrestha, S., & Maharjan, J. (2015). Alkaline thermostable pectinase enzyme from *Aspergillus niger* strain MCAS2 isolated from Manaslu Conservation Area, Gorkha, Nepal. *Springer Plus*, *4*(1), 488. https://doi.org/10.1186/s40064-015-1286-y

Klich, M. A. (2002). *Identification of common Aspergillus species*. Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

Lamounier, K. F. R., Rodrigues, P. O., Pasquini, D., & Baffi, M. A. (2018). Saccharification of Sugarcane Bagasse Using an Enzymatic Extract Produced by *Aspergillus fumigatus*. *Journal of Renewable Materials*, *6*(2), 169–175. https://doi.org/10.7569/JRM.2017.634151

Li, S., Yang, X., Yang, S., Zhu, M., & Wang, X. (2012). Technology Prospecting On Enzymes: Application, Marketing and Engineering. *Computational and Structural Biotechnology Journal*, *2*(3), e201209017. https://doi.org/10.5936/csbj.201209017

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *The Journal of Biological Chemistry*, *193*(1), 265–275.

Madamwar, D., Patel, S., & Parikh, H. (1989). Solid state fermentation for cellulases and β-glucosidase production by *Aspergillus niger*. *Journal of Fermentation and Bioengineering*, *67*(6), 424–426. https://doi.org/10.1016/0922-338X(89)90150-5

Majumdar, D. R., Singh, R., Dondilkar, S., Shaikh, N., Pawale, G., Shinde, P., & Sakate, P. (2018). Enzyme array from thermophilic fungal isolate—RSND. *World Journal of Pharmaceutical Research*, *7*(4), 60–70.

Martins, E. D. S., Leite, R. S. R., Da Silva, R., & Gomes, E. (2012). Production and characterization of polygalacturonase from thermophilic *Thermoascus aurantiacus* on submerged fermentation. *Annals of Microbiology*, *62*(3), 1199–1205. https://doi.org/10.1007/s13213-011-0360-0

Munir, M., Abdullah, R., Haq, I., Kaleem, A., & Iqtedar, M. (2019). Isolation and identification of multi stress tolerant polygalacturonase producing fungi from various fruits. *The Journal of Animal & Plant Sciences*, *29*(3), 825–832.

Oumer, O. J. (2017). Pectinase: Substrate, Production and their Biotechnological Applications. *International Journal of Environment, Agriculture and Biotechnology*, *2*(3), 1007–1014. https://doi.org/10.22161/ijeab/2.3.1

Paolo, D., Bianchi, G., Scalzo, R. L., Morelli, C. F., Rabuffetti, M., & Speranza, G. (2018). The Chemistry behind Tomato Quality. *Natural Product Communications*, *13*(9), 1934578X1801300. https://doi.org/10.1177/1934578X1801300927

Pedrolli, D. B., Monteiro, A. C., Gomes, E., & Carmona, E. C. (2009). Pectin and Pectinases: Production, Characterization and Industrial Application of Microbial Pectinolytic Enzymes. *The Open Biotechnology Journal*, *3*(1), 9–18. https://doi.org/10.2174/1874070700903010009

Pitt, J. I., & Hocking, A. D. (2009). *Fungi and Food Spoilage*. Springer US. https://doi.org/10.1007/978-0-387-92207-2

Rahman, Md. S., Choi, Y. S., Kim, Y. K., Park, C., & Yoo, J. C. (2019). Production of Novel Polygalacturonase from *Bacillus paralicheniformis* CBS32 and Application to Depolymerization of Ramie Fiber. *Polymers*, *11*(9), 1525. https://doi.org/10.3390/polym11091525

Ramesh, A., Harani Devi, P., Chattopadhyay, S., & Kavitha, M. (2020). Commercial Applications of Microbial Enzymes. In N. K. Arora, J. Mishra, & V. Mishra (Eds.), *Microbial Enzymes: Roles and Applications in Industries* (Vol. 11, pp. 137–184). Springer Singapore. https://doi.org/10.1007/978-981-15-1710-5_6

Ramos-Ibarra, J., Miramontes, C., Arias, A., Arriola, E., Guatemala, G., & Corona-González, R. (2017). Production of hydrolytic enzymes by solid-state fermentation with new fungal strains using orange by-products. *Revista Mexicana de Ingeniería Química*, *16*(1), 19–31.

Raper, K., & Fennell, D. (1965). *The Genus Aspergillus*. Williams & Wilkins.

Raveendran, S., Parameswaran, B., Ummalyma, S. B., Abraham, A., Mathew, A. K., Madhavan, A., Rebello, S., & Pandey, A. (2018). Applications of Microbial Enzymes in Food Industry. *Food Technology and Biotechnology*, *56*(1), 16–30. https://doi.org/10.17113/ftb.56.01.18.5491

Ravi, K., & Raghu, R. (2017). Isolation and Screening of Industrially Important Polygalcturonase producing Fungi from the Mangrove Soils of Krishna District Andhra Pradesh. *International Journal of Agricultural Sciences*, *9*(19), 4193–4195.

Reddy, P., & Sreeramulu, A. (2012). Isolation, identification and screening of pectinolytic fungi from different soil samples of Chittoor district. *International Journal of Life Sciences Biotechnology and Pharma Research*, *1*(3), 186–193.

Ribeiro, D. S., Henrique, S. M. B., Oliveira, L. S., Macedo, G. A., & Fleuri, L. F. (2010). Enzymes in juice processing: A review. *International Journal of Food Science & Technology*, *45*(4), 635–641. https://doi.org/10.1111/j.1365-2621.2010.02177.x

Samal, I., Bhoi, T. K., Majhi, P. K., Murmu, S., Pradhan, A. K., et al. (2023). Combatting insects mediated biotic stress through plant associated endophytic entomopathogenic fungi in horticultural crops. *Frontiers in Plant Science*, *13*, 1098673. https://doi.org/10.3389/fpls.2022.1098673

Samson, R., Houbraken, J., Thrane, U., Frisvad, J., & Anderson, B. (2010). *Food and indoor fungi: Second Edition. (2nd ed.)* CBS-KNAW Fungal Biodiversity Centre. C B S Laboratory Manual Series No. 2, CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands

Satapathy, S., Soren, J. P., Mondal, K. C., Srivastava, S., Pradhan, C., Sahoo, S. L., Thatoi, H., & Rout, J. R. (2021). Industrially relevant pectinase production from *Aspergillus parvisclerotigenus* KX928754 using apple pomace as the promising substrate. *Journal of Taibah University for Science*, *15*(1), 347–356. https://doi.org/10.1080/16583655.2021.1978833

Shamly, V., Kali, A., Srirangaraj, S., & Umadevi, S. (2014). Comparison of Microscopic Morphology of Fungi Using Lactophenol Cotton Blue (LPCB), Iodine Glycerol and Congo Red Formaldehyde Staining. *Journal of Clinical and Diagnostic Research*, *8*(7), DL01-02. https://doi.org/10.7860/JCDR/2014/8521.4535

Siddiqui, Mohd. A., Pande, V., & Arif, M. (2012). Production, Purification, and Characterization of Polygalacturonase from *Rhizomucor pusillus* Isolated from Decomposting Orange Peels. *Enzyme Research*, *2012*, 1–8. https://doi.org/10.1155/2012/138634

Soares, I., Távora, Z., Barcelos, R., & Baroni, S. (2012). Microorganism-produced enzymes in the food industry. In B. Valdez, M. Schorr, & R. Zlatev (Eds.), *Scientific, health and social aspects of the food industry* (pp. 83–94). IntechOpen.

Tai, E.S., Hsieh, P.C., & Sheu, S.C. (2013). Purification and Characterization of Polygalacturonase from Screened *Aspergillus tubingensis* for Coffee Processing. *Food Science and Technology Research*, *19*(5), 813–818. https://doi.org/10.3136/fstr.19.813

Thakur, A., Pahwa, R., Singh, S., & Gupta, R. (2010). Production, Purification, and Characterization of Polygalacturonase from *Mucor circinelloides* ITCC 6025. *Enzyme Research*, *2010*, 1–7. https://doi.org/10.4061/2010/170549

Wang, S., Lian, Z., Wang, L., Yang, X., & Liu, Y. (2015). Preliminary investigations on a polygalacturonase from Aspergillus fumigatus in Chinese Pu'er tea fermentation. *Bioresources and Bioprocessing*, *2*(1), 33. https://doi.org/10.1186/s40643-015-0061-9

Yoon, L. W., Ang, T. N., Ngoh, G. C., & Chua, A. S. M. (2014). Fungal solid-state fermentation and various methods of enhancement in cellulase production. *Biomass and Bioenergy*, *67*, 319–338. https://doi.org/10.1016/j.biombioe.2014.05.013

Zhang, S., Amin, F., Xiong, M., Bhatti, H. N., & Bilal, M. (2021). High-yield intracellular production of an exo-polygalacturonase enzyme via heterologous expression of *Penicillium notatum* gene in *Saccharomyces cerevisiae*. *International Food Research Journal*, *28*(4), 664–671. https://doi.org/10.47836/ifrj.28.4.03