*British Biotechnology Journal*
*12(1): 1-10, 2016, Article no.BBJ.23632*
*ISSN: 2231–2927, NLM ID: 101616695*

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# Purification and Characterization of Pectin Methylesterase Produced in Solid State Fermentation by *Aspergillus tubingensis*

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***Authors' contributions***

*This work was carried out in collaboration between all authors. Author MKP carried the experimental work and wrote the first draft of the manuscript. Author AN designed the study, supervised the experimental work, prepared the figures. Author SN also designed the experiment, checked and corrected the first draft of the manuscript. Author AK designed the experimental work, managed the literature search and finally corrected the entire manuscript. All authors read and approved the final manuscript.*

***Article Information***

DOI: 10.9734/BBJ/2016/23632
Editor(s):
(1) Miguel Cerqueira, Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho, Portugal.
Reviewers:
(1) Eduardo da Silva Martins, Minas Gerais State University, Brazil.
(2) Maria Gabriela Bello Koblitz, Federal University of the State of Rio de Janeiro, Brazil.
(3) Danielle Biscaro Pedrolli, Sao Paulo State University, Brazil.
Complete Peer review History: http://sciencedomain.org/review-history/13038

***Original Research Article***

***Received 11th December 2015***
***Accepted 12th January 2016***
***Published 21st January 2016***

## ABSTRACT

**Aim:** Purification and characterization of pectin methylesterase produced by *Aspergillus tubingensis* in solid state fermentation.
**Study Design:** Pectin methylesterase enzyme produced by *A. tubingensis* was extracted from the fermented solid medium and purified using chromatographic techniques. The purified enzyme was characterized for physico-chemical and kinetic properties.
**Place and Duration of Study:** Experiments were performed at the School of Biotechnology, Devi Ahilya University, Indore, INDIA and Maharaja Ranjit Singh College of Professional Sciences, Indore, INDIA, between October, 2014 and August, 2015.

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**Methodology:** The enzyme was extracted and purified using ammonium sulphate fractionation, ion exchange chromatography (IEC) using CM- cellulose and gel filtration chromatography (GFC) using Sephadex G-100. The molecular weight of the purified enzyme was determined using native polyacrylamide gel electrophoresis (Native PAGE) and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE). The purified enzyme was characterized to determine the pH and temperature optima. Thermostability, pH stability and substrate kinetics were studied for purified pectin methylesterase.

**Results:** The acidic pectin methylesterase of *Aspergillus tubingensis* was purified to 20.3 fold with a 47.7% recovery through IEC on CM- cellulose and GFC using Sephadex G-100. The purified enzyme had a specific activity, 112.6 U/mg. The SDS-PAGE revealed that the enzyme was monomeric with a molecular weight of 45.7 kDa. The optimum pH and temperature were 4.6 and 50°C, respectively. This enzyme was stable over a wide pH range (3.0–8.0) and at relatively high temperature at 50°C for 1 h. The K*m* and V*max* values of pectin methylesterase towards citrus pectin were 33.3 mg/l and 251.2 µmol/ml/min, respectively. In addition, the enzyme activity increased by about 16% in the presence of 5 mM $Mg^{2+}$.

**Conclusion:** The pectin methylesterase enzyme of *A. tubingensis* has been purified up to homogeneity and found to be monomeric on SDS-PAGE. Enzyme characterization revealed that purified enzyme worked optimally in acidic conditions and was stable at wider pH range.

*Keywords: Pectin methylesterase; purification; ion exchange chromatography; gel filtration chromatography; characterization; molecular weight.*

## 1. INTRODUCTION

Pectinolytic enzymes or pectinases are a heterogeneous group of enzymes that hydrolyze the pectic substances present in plants [1,2]. Pectin is a methoxylated galacturonic acid polymer that provides integrity and rigidity to plant tissues [3]. It is sequentially hydrolyzed by pectin methylesterase and polygalacturonase enzymes during cell growth [4]. Pectin methylesterase (pectinesterase, PME, EC 3.1.1.11) catalyzes the de-esterification of pectin, yielding methanol and the pectin with a lower degree of esterification called pectate [5,6]. It acts as a substrate for polygalacturonase (PG, EC 3.2.1.15), an enzyme that depolymerizes the polysaccharide chain [7]. Pectin methylesterase is produced by plants [8,9], plant parasites [10] and many different plant pathogenic and saprophytic organisms including bacteria and fungi [11,12]. Microbial pectinolytic enzymes are among the most important industrial enzymes and are of great significance with wide range of applications in fruit juice extraction and clarification, textile processing, degumming of plant bast fibers, treatment of pectic waste waters, paper making and coffee and tea fermentations [2,13].

Fungal pectin methylesterase (PME) plays a key role in the propagation of plant pathogens [5]. PMEs are found in phytopathogenic fungi, such as *Aspergillus* sp. [14-16], *Botrytis* sp. [17], and *Fusarium sp.* [18,19]. According to Glinka and Liao [18], only a limited number of PMEs have been purified and characterized in detail. In general, most fungal PMEs have optimal pH values between 4 and 6 [2,18]. PMEs are medium sized enzymes whose molecular weight varies between 25-50 kDa. Medium sized fungal PME have also been reported from *Fusarium asiaticum* [18], *Aspergillus aculeatus* [15], *Aspergillus niger* [20,21] *Aspergillus oryzae* [22], *Aspergillus japonicus* [23], *Fusarium oxysporum* [24] and *Botrytis cinerea* [25]. In the present study, PME produced by locally isolated *A. tubingensis* has been purified using ion exchange and gel filtration chromatography, and purified enzyme has been characterized.

## 2. MATERIALS AND METHODS

### 2.1 Materials

Pectin (degree of esterification: 90-95%), D-galacturonic acid were purchased from Sigma-Aldrich, USA. Papaya peel was collected from local fruit shops, dried and used. All the other chemicals were of high quality analytical grade procured locally.

### 2.2 Microorganism and Inoculum Preparation

The fungal strain used in this study was locally isolated from soil of fruit processing area and identified as *Aspergillus tubingensis*. This strain

was cultivated on potato dextrose agar (PDA), pH 5.6 and temperature 28°C. It was stored at 4°C.

Inoculum was prepared on PDA slants of *Aspergillus tubingensis*, which were incubated at 28°C for 5 days. Spore suspension (1 x $10^7$ spores /ml) was prepared by adding sterile distilled water on slant. Sterile glass rod was used to scrap the spores. Spores filtered with sterile glass wool were counted using counting chamber and used for inoculation in solid state fermentation medium.

## 2.3 Solid State Fermentation (SSF)

PME production in SSF was carried out using dried papaya peel. The substrate papaya peel was dried naturally in sun light. For SSF, 15 g of papaya peel having mesh size 10 was taken in 250 ml Erlenmeyer flask and initial moisture content 86% (v/w) was maintained with 10% orange peel suspension. The substrate was sterilized in autoclave at 121°C for 20 minutes at 15 psi and made to undergo SSF for 5 days at 30°C by inoculating with 1.5 ml of spore suspension having spore content 1 x $10^7$ spore/ml.

## 2.4 Enzyme Extraction and Assay

After SSF, 20 mM citrate phosphate buffer, pH 4.6 was added in the biomass to get 1:10 (w/v) ratio. Thereafter, 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 x *g* for 30 min in cold condition (0 to 4°C) and the supernatant was used as the crude enzyme.

PME was assayed by continuous spectrophotometric method using citrus pectin as a substrate as described by Vilariño et al. [26] with some modifications. A 3.0 ml reaction mixture contained 2.6 ml of 0.1% citrus pectin pH 4.6 and 0.3 ml of 0.2% bromocresol green, pH 4.6. The reaction was started by adding 0.1 ml of the enzyme preparation and decrease in absorbance was recorded at 617 nm after every 30 seconds. A control was also prepared where enzyme preparation was added after heat denaturation. The fall in pH was equated to galacturonic acid units released as per calibration curve prepared. Galacturonic acid standard (2-20 µmol) was prepared in distilled water and was used to calibrate the fall in pH with increase in galacturonic acid concentration. Temperature was maintained at 50°C. One unit of the enzyme activity was taken as the amount of enzyme that catalyses the release of 1 µmol of galacturonic acid equivalent per minute under the conditions of the enzyme assay.

## 2.5 Protein Determination

Protein concentration was determined using Lowry's method [27] where bovine serum albumin (BSA) was used as the standard. In this method, protein was precipitated by adding trichloroacetic acid (TCA) to a final concentration of 5%. The precipitated protein was centrifuged at 10,000 x *g* for 10 min and washed with 5% TCA to remove any adhering impurities. The pellet was dissolved in water and assayed.

## 2.6 Enzyme Purification

To the crude enzyme (250 ml), 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 addition of ammonium sulfate, suspension was incubated for 3 h in the cold condition to precipitate proteins. Thereafter, it was centrifuged at 10,000 x *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 x *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 for overnight in the cold condition to precipitate proteins. The precipitated proteins were collected by centrifugation at 10,000 x *g* for 30 min in the cold condition. The pellets after each centrifugation were dissolved in citrate phosphate buffer, pH 4.6 and tested for PME enzyme activity. The 60 to 90% fraction which had maximum PME enzyme activity was centrifuged at 10,000 x *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 fractions showing pectin methylesterase activity were pooled and loaded on regenerated CM cellulose column. The flow rate of the column was 0.5 ml /min. The unbound protein was collected by elution with two bed volumes of buffer before starting the gradient and enzyme activity was analyzed in it. The ionically bound enzyme was eluted from the column using 0–1 M

NaCl gradient prepared in same buffer using gradient mixer. The fractions of 5 ml were collected at the flow rate of 2 ml/min. The protein content and the enzyme activity in each fraction were analyzed. The active fractions constituting a single peak were pooled and concentrated by reverse dialysis against solid sucrose using a nitrocellulose membrane (Hi media- 135). The pooled fraction (10 ml) was put in the nitrocellulose membrane and 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 pool was applied to a Sephadex G-100 column equilibrated with 20 mM citrate- phosphate buffer, pH 4.6. The flow rate of the column was 10 ml/ h. The fractions of 2 ml were collected and PME activity and protein concentration in each fraction was estimated. Protein fractions corresponding to PME activity were pooled and concentrated by reverse dialysis.

## 2.7 Electrophoresis

The molecular weight of the purified enzyme was estimated by NATIVE-PAGE (10%) and SDS–PAGE (10%) according to the method of Laemmli [28] using a gel electrophoresis system (Bangalore Genei). Protein bands were stained with coomassie brilliant blue R-250. Molecular mass of purified PME was estimated using standard protein molecular weight markers of known molecular mass 18.4–240 kDa for Native PAGE and 14– 95 kDa for SDS–PAGE.

Zymogram study of PME was performed using pectin acrylamide gel as described by Jiang et al. [29]. The stacking gel of pH 6.8 and separating gel of pH 8.8, both were prepared with 0.1% citrus pectin. Electrophoresis was performed at 4°C for 4 h at a constant voltage of 100 V. After electrophoresis, the gel was washed twice with distilled water. The gel was immersed in assay buffer at 50°C for 45 min and then stained with 0.05% ruthenium red (pH 4.6) for 45 min.

## 2.8 Characterization of Purified Enzyme

### 2.8.1 Effect of pH on enzyme activity and pH stability

The effect of pH on enzyme activity was determined by preparing the reaction mixture in 20 mM citrate- phosphate buffer pH ranging from 3.6 to 5.4 with interval of 0.2. In order to determine the pH stability, the enzyme was pre-incubated in 50 mM sodium-acetate buffer (pH 3.0, 4.0, 5.0, 6.0), 50 mM phosphate buffer (pH 7.0, 8.0) and 50 mM glycine-NaOH buffer (pH 9.0, 10.0) at 4°C for 1 h and then assayed for pectin methylesterase activity with citrus pectin as substrate.

### 2.8.2 Effect of incubation temperature and thermostability

The effect of incubation temperature was determined by incubating enzyme and substrate reaction mixture at temperature ranging from 30 to 70°C with difference of 5°C and enzyme assay was performed as mentioned above.

The thermal stability was investigated by incubating the purified enzyme alone at temperature between 30 to 70°C with an interval of 5°C for 1 h. At each temperature, the enzyme activity was determined using optimized conditions of pH and temperature.

### 2.8.3 Effect of substrate concentration

The *K*m and *V*max values of the purified enzyme were determined with citrus pectin prepared in distilled water (pH 4.6). To determine the optimum substrate concentration, different concentrations of substrate from 1 to 10 mg/ l were used for assay at optimum pH 4.6 and temperature 50°C. *K*m and *V*max was determined using double reciprocal plot.

### 2.8.4 Effect of metal ions

The effect of metals on the activity of the purified PME was determined in the presence of 2 mM, 5 mM and 10 mM of $Na^{+}$, $Mg^{2+}$, $K^{+}$, $Ni^{2+}$, $Mn^{2+}$, $Ca^{2+}$, $Co^{2+}$ in the reaction mixture. The effects of EDTA, SDS, and Triton X-100 on the enzymatic activity were determined at concentration 10 mM, 0.1% (w/v), and 0.05% (v/v), respectively. The metal ions and chemicals were incorporated in the reaction mixture and assay was carried out at 50°C for 20 min. Activity was determined as described earlier and expressed as a percentage of the activity obtained in the absence of the metal cation and chemical agent. The reaction mixture without metal ion or chemical was used as control.

## 3. RESULTS AND DISCUSSION

## 3.1 Enzyme Purification

The crude enzyme obtained after 120 h fermentation (unpublished data) by *A.*

*tubingensis* was subjected to ammonium sulphate fractionation and maximum protein precipitated in 60-90% fraction. The specific activity increased to 9.7 U/mg with 88.5% recovery and 1.7 fold purification (Table 1). This step was performed as a purification step to remove unwanted substances and proteins, as well as a concentration step. This precipitation step was efficient because it reduced the total protein amount by nearly 50.4%. The dialyzed enzyme preparation was fractionated on a CM-Cellulose column and the elution profile is shown in Fig. 1.

The unbound enzyme along with most of the contaminating proteins came out in the form of colored fractions. No PME activity was determined in these unbound fractions. The activity eluted out as a single peak with a linear salt gradient of 0–1 M. The activity fractions were pooled and concentrated by nitrocellulose membrane. The concentrated pool was fractionated separately by gel exclusion chromatography on a Sephadex G-100 superfine column for the final purification step. In this chromatography, single peak of enzyme activity was observed (Fig. 2). The enzyme was purified about 20.3 fold with an increase in specific activity to 112.6 U/mg giving a yield of 47.7%. There was no polygalacturonase activity observed in the purified enzyme.

Maldonado et al. [30] reported purification of PME from *Aspergillus niger* and showed 88 fold purification using Sephadex G-150 and DEAE Sephacel A chromatography. Forster and Rasched [31] purified PME produced by fungus *Phytophthora infestans* with 12% recovery using CM cellulose and Sephacryl S-200. Jiang et al. [29] reported 28% yield with 342 U/mg specific activity of *Aspergillus niger* PME.

NATIVE PAGE analysis and Zymogram study revealed that pectin methylesterase is the predominant protein in the purified preparation (Figs. 3a,b). In SDS PAGE study, enzyme molecular mass was estimated to be 45.7 kDa on 10 % separating gel when stained by coomassie brilliant blue R-250 (Fig.4) which is in accordance with earlier reports of PME molecular weight isolated from other species of *Aspergillus* [20,23]. The molecular weight of PME with native PAGE was found nearly 51.3 kDa indicating that it is a monomeric protein. Difference in the values with SDS PAGE and Native PAGE is because native PAGE is not much sensitive technique for calculation of molecular weight where separation is on the basis of size as well as charge.

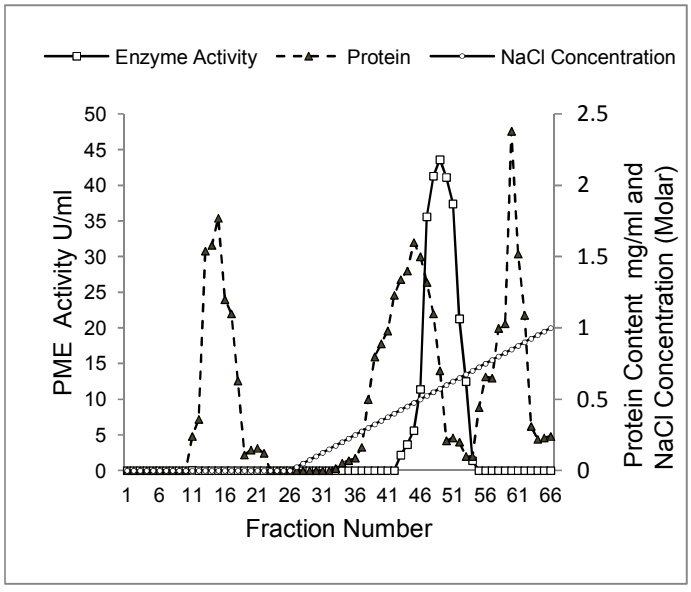


**Fig. 1. Elution profile of bound and unbound fractions on CM cellulose column. The column was equilibrated with 20 mM citrate phosphate buffer (pH 4.6) and eluted with a step gradient of NaCl in the same buffer. Fractions (5 ml) were collected at a flow rate 2 ml per min**

**Table 1. Summary of purification of pectin methylesterase produced by *Aspergillus tubingensis* using solid state fermentation**

| Purification steps | Enzyme units | Total protein (mg) | Specific activity (U/mg) | Fold purification | Yield % |
|---|---|---|---|---|---|
| Crude | 1949 | 358.2 | 5.4 | 1 | 100 |
| Ammonium Sulphate | 1725.9 | 177.7 | 9.7 | 1.7 | 88.5 |
| CM Cellulose | 1285.5 | 48.8 | 26.3 | 4.8 | 65.9 |
| Sephadex G-100 | 930.4 | 8.2 | 112.6 | 20.3 | 47.7 |

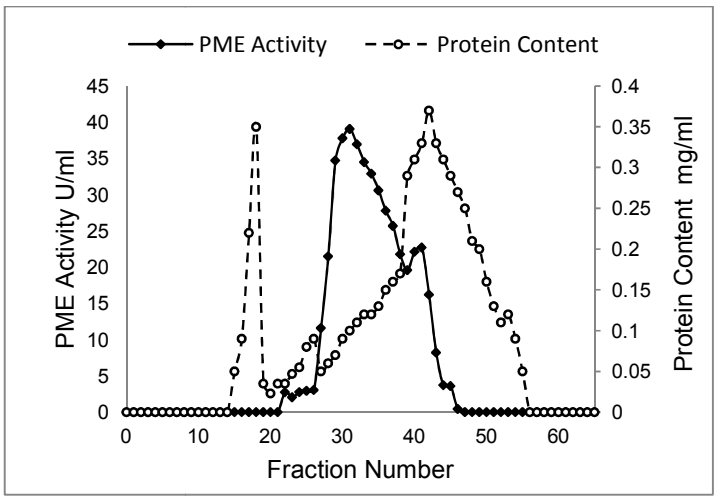


**Fig. 2. Elution profile of PME on Sephadex G-100 gel filtration chromatography. The column was equilibrated with 20 mM citrate phosphate buffer. Fractions of 2 ml were collected at a flow rate of 10 ml/ h**

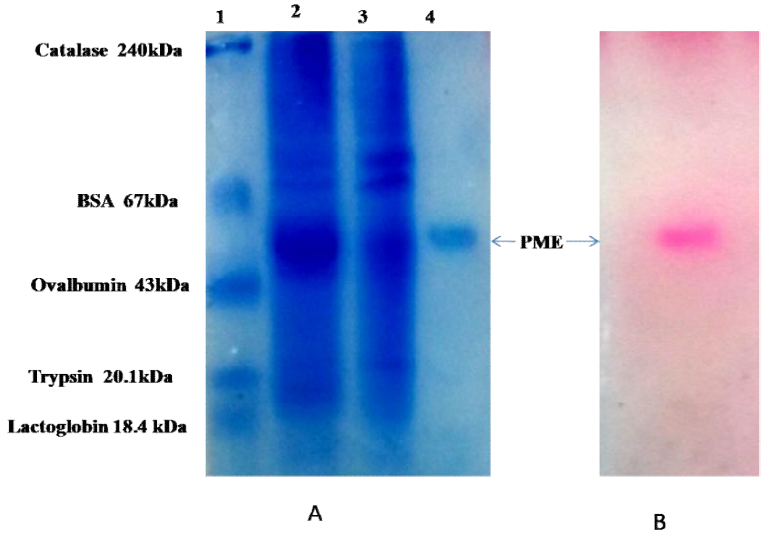


**Fig. 3. (A) Native PAGE of *A. tubingensis* PME (1) molecular weight marker (2) Crude sample (3) PME sample after ion exchange chromatography (4) PME after Sephadex G-100 gel chromatography (B) Activity staining of *A. tubingensis* PME using pectin polyacrylamide gel**

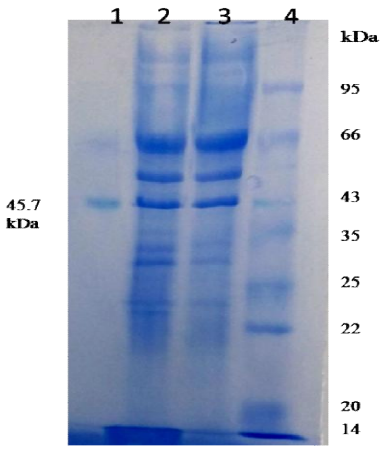


**Fig. 4. SDS-PAGE of *A. tubingensis* PME. (1) PME after Sephadex G-100 gel chromatography (2) PME after CM cellulose chromatography (3) Crude sample of PME (4) Molecular mass standards**

## 3.2 Enzyme Characterization

### 3.2.1 Effect of pH and pH stability

The optimum PME activity was found at pH 4.6 (Fig. 5 a). The relative activity of PME at pH 4.0 and 5.0 was 30.3% and 47%, respectively. Similar results were shown by Maldonado et al. [30]. Higher pH 6.5 and 7.6 was shown by Glinka and Liao [18] and Markovic et al. [32] for PME of *Fusarium asiaticum* and *Trichoderma reesei,* respectively. The purified enzyme was reasonably active between pH 3 and 8. At pH 3.0, it showed 66% of the residual activity. At higher pH 8, PME residual activity was 30.8%. The highest stability of PME was found at pH 5.0 where 100% residual activity was observed (Fig. 5b).

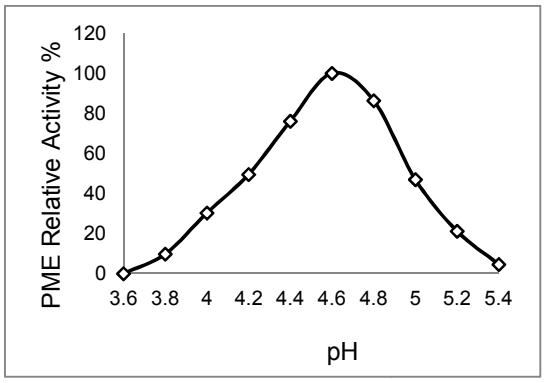


**Fig. 5. (a) Effect of pH on activity of pectin methylesterase from *A. tubingensis***

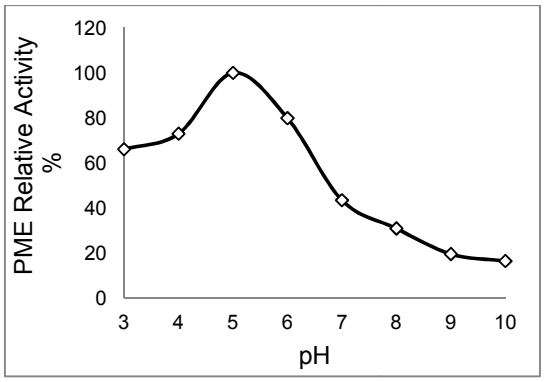


**Fig. 5. (b) Effect of pH on stability of PME. Purified PME was incubated for 1 h with different pH ranging from 3-10 at 4°C**

### 3.2.2 Effect of temperature and thermostability

Maximum activity of PME with 0.1% citrus pectin was found at 50°C with two half temperature optima at 60°C and 40°C (Fig. 6a). Jiang et al. [29] also reported maximum activity of *A. niger* PME at 50°C but total inactivation of the enzyme at 60°C. The thermostability results indicated that present PME was quite stable up to 1 h at 50°C. However, enzyme was fully stable only for 15 min at 60°C. It lost about 20% enzyme activity after incubation at 60°C for 30 min (Fig. 6b). Dirix et al. [33] reported loss of nearly 40% of *A. aculeatus* PME enzyme activity after incubation at 50°C for 15 min and loss of more than 95% enzyme activity after incubation at 55°C for 15 min.

### 3.2.3 Enzyme Kinetics

The K*m* and V*max* values of the PME were determined using Lineweaver–Burk plot (Fig. 7). The effect of increasing substrate concentration on the enzymatic reaction rate followed a typical Michaelis–Menten kinetics using citrus pectin as the substrate. The K*m* and V*max* values of PME were calculated to be 33.3 mg/l and 251.2 U/mg protein, respectively, at pH 4.6 and 50°C.

### 3.2.4 Effect of metal ions, chelating agent and detergent

As shown in Table 2, the PME activity was partly inhibited by $K^+$, $Ni^{2+}$, $Ca^{2+}$, and completely inhibited by $Co^{2+}$. These results are different from those of other previously reported PME, where PME activity was inhibited by $Mg^{2+}$ [30]. The difference may have been caused by the amino

acid sequence of PME. The effect of chemicals on PME revealed that PME was sensitive to 5 mM EDTA, 0.1% (w/v) SDS, and 0.05% Triton X-100 (Table 3). Jiang et al. [29] reported inhibitory effects of cations such as $K^+$, $Mg^{2+}$, $Ni^{2+}$, $Mn^{2+}$ and $Co^{2+}$, however, no effect of $Na^+$ was observed.

**Table 2. Effect of various metal ions at 2 mM 5 mM and 10 mM concentration on PME activity**

| Reagent | Relative activity (%) of PME | | |
|---|---|---|---|
| | 2 mM | 5 mM | 10 mM |
| Control | 100 | 100 | 100 |
| $Na^+$ (NaCl) | 100 | 100 | 100 |
| $Mg^{2+}$ ($MgCl_2$) | 93.6 | 116.8 | 116.3 |
| $K^+$ (KCl) | 91.7 | 87.4 | 76.3 |
| $Ni^{2+}$ ($NiCl_2$) | 45.7 | 35.7 | 13.5 |
| $Mn^{2+}$ ($MnCl_2$) | 8.6 | 0 | 0 |
| $Ca^{2+}$ ($CaCl_2$) | 100 | 99.4 | 99.3 |
| $Co^{2+}$ ($CoCl_2$) | 0 | 0 | 0 |

**Table 3. Effect of different chemicals on PME activity**

| Chemical | Relative activity % |
|---|---|
| EDTA (5mM) | 22.5 |
| SDS (0.1% w/v) | 64.8 |
| Triton X 100 (0.05% v/v) | 35.02 |

Our results on one side indicated no effect of most of the tested cations except $Mg^{2+}$ whose presence at 5 mM concentration stimulated the enzyme activity about 16%, whereas on other side, chelating agent, EDTA inhibited PME activity up to nearly 77% when added at 5 mM concentration. It looks that EDTA changes conformation of the enzyme molecule and /or there is some bound cation essential for enzyme activity.

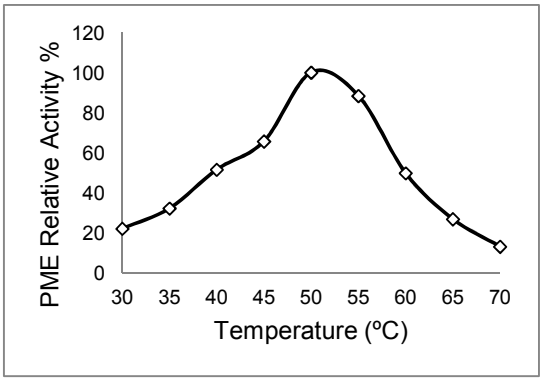


**Fig. 6. (a) Effect of incubation temperature on activity of PME from *A. tubingensis***

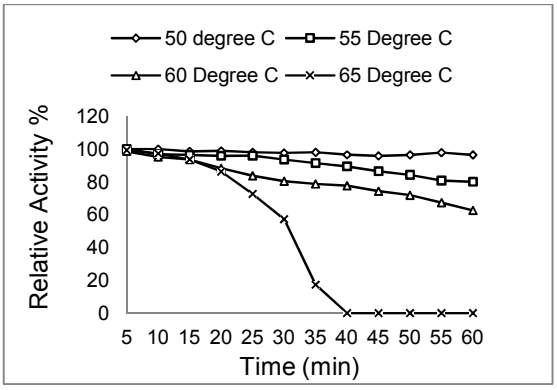


**Fig. 6. (b) Thermostability of purified PME at different temperatures from 50-65°C for different time intervals**

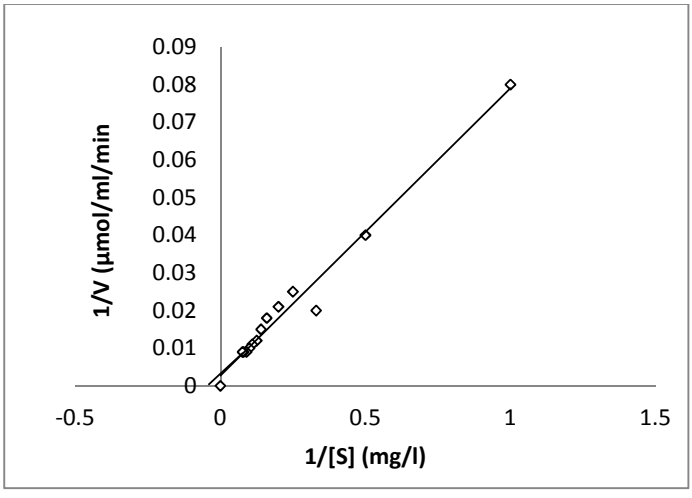


**Fig. 7. Lineweaver–Burk plot for PME- catalyzed reaction. Enzyme reaction was carried out at 50ºC, pH 4.6 and citrus pectin was used as substrate**

## 4. CONCLUSION

The results presented in this paper demonstrated that under the employed SSF fermentation conditions PME was produced by *A. tubingensis*. Single form of PME has been found in *A. tubingensis*. PME with the molecular weight of 45.7 kDa was purified to near homogeneity. The purified PME was stable at pH from 4 to 8 and temperature at 50-60°C for 1 h. This research provides the basis for future applications of PME in food industries.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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