Vol. 20, No. 2 (2021) 1029-1045
Revista Mexicana de Ingeniería Química

Kinetic and thermodynamic insight of a polygalacturonase: A biocatalyst for industrial fruit juice clarification

Conocimiento cinético y termodinámico de una poligalacturonasa: Un biocatalizador para la clarificación industrial de jugos de frutas

A. Nawaz*, M. Sameer, F. Akram, S.F. Tahir, Y. Arshad, I.U. Haq, H. Mukhtar

Institute of Industrial Biotechnology,Government College University, Lahore, 54000 Pakistan.

Received: February, 2021; Accepted: April 16, 2021

Abstract

In this study, complete purification, characterization, and immobilization of polygalacturonase (pectinase) from *Penicillium notatum* were carried out to achieve an economical and suitable alternative for industrial fruit juice clarification. Biosynthesis of polygalacturonase was carried out under pre-optimized conditions employing solid-state fermentation at a pilot scale using wheat bran.Polygalacturonase was produced economically by using agro-industrial residues.The enzyme was subjected to a series of steps for purification including ion-exchange chromatography. After that, the purified enzyme was characterized and its kinetics and thermodynamic parameters along with the effect of immobilization on its performance were studied. Finally, the purified acidic enzyme was tested for its clarifying abilities on fresh apple juice. Purification fold of 2.98 was attained with an increased specific activity of 256U/mg. Purified polygalacturonase showed a molecular weight of 38 kDa, optimum temperature of 50 ºC, optimum pH of 5, 50% stability at 50 ºC, and 84% stability at pH 5. The “Vmax” and “Km” of the enzyme were evaluated to be 250U/mg and 0.11mg/mL, respectively for hydrolyzing pectin. From the Arrhenius plot, activation energy (E_a), enthalpy of activation (ΔH), and entropy of activation (ΔS) were found to be 6.35 KJ/mol, 3.67 KJ/mol, and -1.1KJ/mol, respectively. Among metal ions, most of the tested organic solvent and inhibitors inhibited the activity. Nano emulsion-based pectinase exhibited better stability. The enzyme was found to be an effective agent for the clarification of fresh apple juice.

Keywords: Catalysis, immobilization, characterization, stability, purity, pectin.

Resumen

En este estudio se realizó la purificación completa, caracterización e inmovilización de poligalacturonasa (pectinasa) de *Penicillium notatum* para lograr una alternativa económica y adecuada para la clarificación industrial de jugos de frutas. La biosíntesis de poligalacturonasa se llevó a cabo en condiciones optimizadas previamente empleando fermentación en estado sólido a escala piloto utilizando salvado de trigo. La poligalacturonasa se produjo de forma económica mediante el uso de residuos agroindustriales. La enzima se sometió a una serie de pasos para su purificación, incluida la cromatografía de intercambio iónico. Posteriormente, se caracterizó la enzima purificada y se estudiaron su cinética y parámetros termodinámicos junto con el efecto de la inmovilización sobre su desempeño. Finalmente, se probó la enzima ácida purificada para determinar su capacidad clarificadora en jugo de manzana fresco. Se logró un pliegue de purificación de 2,98 con una actividad específica aumentada de 256 U / mg. La poligalacturonasa purificada mostró un peso molecular de 38 kDa, una temperatura óptima de 50 ° C, un pH óptimo de 5, 50% de estabilidad a 50 ° C y 84% de estabilidad a pH 5. La "Vmax" y "Km" de la enzima fueron evaluados en 250U / mg y 0,11 mg / ml, respectivamente, para hidrolizar la pectina. A partir de la gráfica de Arrhenius, se encontró que la energía de activación (E_a), la entalpía de activación (ΔH) y la entropía de activación (ΔS) eran 6.35 KJ / mol, 3.67 KJ / mol y -1.1KJ / mol, respectivamente. Entre los iones metálicos, la mayoría de los inhibidores y disolventes orgánicos probados inhibieron la actividad. La pectinasa basada en nano emulsión mostró una mejor estabilidad. Se descubrió que la enzima es un agente eficaz para la clarificación del jugo de manzana fresco.

Palabras clave: Catálisis, inmovilización, caracterización, estabilidad, pureza, pectina.

* *Corresponding author. E-mail*: ali.nawaz@gcu.edu.pk
https://doi.org/10.24275/rmiq/Bio2355
ISSN:1665-2738, *issn-e*: 2395-8472

1 Introduction

Pectins, which provide rigidity to plant cells, are complex polysaccharides composed of branched neutral sugar side chains and a backbone consisting of methyl esterified α- (1, 4) linked homogalacturonic acid (Usha *et al*., 2014; Kumar, 2015).Pectinases (E.C.3.2.1.15) are a group of enzymes that hydrolyzes the pectic substances by cleaving the glycosidic bond of galacturonic acid (Ubani *et al*., 2015).Pectinases are classified as polysaccharide hydrolases, polysaccharide lyase, carbohydrate esterases, pectin lyases (EC 4.2.2.10), pectate lyases (EC 4.2.2.2), and pectin methyl esterases (EC 3.1.11)based on the mechanism used to attack the galacturonan backbone (Mohandas *et al*., 2018). In the family of pectinolytic enzymes, polygalacturonase (PG) is the most studied class. In food processing industries, it is widely used for the extraction as well as clarification of acidic fruit juices. Pectinases have a large number of applications in the commercial sector which include the textile industry, plant fibers processing, tea and coffee industry, wastewater treatment, oil extraction industry, etc. (Ma *et al*., 2016).

Pectinases can be extracted from both plants and microorganisms. The main sources of these enzymes are fruits and vegetables (Khadija *et al*., 2016). Among microbial sources, *Aspergillus niger, Aspergillus oryzae, Penicillium restrictum, Penicillium expansum, Trichoderma viridae, Mucor piriformis, Chryseobacterium indologens, Bacillus subtilis, Bacillus cereus, Bacillus licheniformis*, and *Actinomycetes* are of prime importance (Bhardwaj *et al*., 2017).Microbial production of pectinases is preferred over plant sources because enzyme produced from microbial source is more controllable and predictable as pH is within neutral range, low production cost, controllability of enzyme content, less space required for enzyme cultivation, and rapid growth (Sindhu *et al*., 2017). Commercially, these enzymes are obtained from fungi because they can be cultured easily (Javed *et al*., 2018).

Pectinase is usually required in a purified form with an understanding of the structure and functional mechanisms. Based on various characteristics of an enzyme such as size, charge, and solubility, several thousand proteins have been purified (Dange and Harke, 2018). For industrial use, the enzyme should be essentially purified and characterized. Purification is also significant to make the enzyme nonpathogenic for human consumption. In purification after each step, protein concentration is calculated (Obafemi *et al*., 2019). Some of the best-known methods for purification are ammonium sulfate precipitation (Bharadwaj and Udupa, 2019), dialysis, and ion-exchange chromatography. Moreover, characterization of protein is also essential as it not only differentiates between complex components of an enzyme, but also regulates enzyme synthesis, conditions of optimum activity, and mechanism of substrate degradation (Okonji *et al*., 2019).

Pectin and other polysaccharides present in the fruit affect the commercial value of juice as they lead to colloid formation and fouling. For clarification of juices,the purified pectinase enzyme is used as it reduces the viscosity and cluster formation of juices and has high commercial value (Anand *et al*., 2017). A part form pectinase, other enzymes are also being used for the clarification of fruit juices including carbohydrase, lipase, tannase and naringinase, etc. (Singh *et al*., 2019). As polygalacturonase has wide industrial applications, it can be immobilized using calcium alginate and nanoemulsion to make expensive enzymes economically viable (Cardoso *et al*., 2019; Ahmed *et al*., 2019).

To meet the industrial needs pectinase produced should be economical and the need is to improve yield without enhancing the production cost. For this purpose, in this study agro-industrial residues such as wheat bran, sugarcane bagasse, etc. are used (Oumer and Abate, 2018).Penicillium is suitable for the production of pectinase due to production of more biomass, higher yield, low proteolysis and stability of enzyme at low temperature. This work is significant as fungi are excellent producers of pectic enzymes and the optimum pH of fungal enzymes is very close to the pH of many fruit juices, which range from pH 3.0 to 5.5. Preparations containing pectin-degrading enzymes used in the food industry are also derived from fungi (Siddique *et al*., 2012). Considering the above facts, the present study reports the purification, characterization, and immobilization of polygalacturonase from *P. notatum* and its application in fruit juice clarification.

2 Materials and methods

2.1 Microorganism

A fungal strain *Penicillium notatum* (MAIIB-33) was used in this study for the production of polygalacturonase enzyme (PG), which was obtained from the culture bank of the Institute of Industrial Biotechnology, GC University Lahore, Pakistan.

2.2 Enzyme production and optimization of carbon sources

The pilot-scale production of PG was done using a tray bioreactor (Samwon Eng, SU-105IP, Korea) under optimum fermentation conditions i.e. 30 ºC temperature, pH 5.5, 150 rpm agitation, and 5 days incubation (Patidar *et al*., 2018). The Tray bioreactor included three trays located at an equal distance having humidity and temperature controllers. Diluent was contained in the bottom tray and was circulated by a peristaltic pump on the solid bed which was spread on perforated trays. Circulation of the liquid medium was carried out until desired humidity was achieved. An incandescent bulb provided the required temperature and uniform air was provided by the small fans inside the bioreactor. The fermentation medium was composed of pectin (180g) and wheat bran (10kg) moistened with 3L of distilled water as diluent [$(NH_4)_2SO_4$ (1200g/L), $MgSO_4$ (300g/L), KH_2PO_4 (600g/L), FeH_4O_6S (0.189g/L), $ZnSO_4$ (0.189g/L), $MnSO_4$ (0.03g/L)] (Sandri and Silveira, 2018). Enzyme production in culture media was induced by using different agro-industrial residues such as wheat bran, sugarcane bagasse, rice straw, orange peel, lemon peel, and apple peel. Pectin and glucose were used as a positive and negative control, respectively (Pagnonceli *et al*., 2019). After incubation, the substrate was mixed in phosphate buffer (pH 6) for extraction and the content was centrifuged at 6000rpm for 10 minutes.

2.3 Purification

The crude extract obtained after extraction was precipitated using ammonium sulfate with 30-80% saturation. Then dialysis was done to remove unwanted salts using phosphate buffer for 24-48h. The enzyme was purified to isoelectric homogeneity through anion-exchange chromatography (Bio-Rad BioLogic LP, USA) by utilizing the Bio-scale Mini MacroPrep DEAE cartridge column using 50mM Tris-Cl (pH 8) as an equilibrating buffer and 50 mM Tris-Cl with 0.1M NaCl as elution buffer. For identification of protein of interest, enzyme assay of both bound and unbound fractions was done and the percentage purity of polygalacturonase was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Cheng *et al*., 2016).

2.4 Enzyme assay

The activity of Polygalacturonase was determined by evaluating the liberation of reducing sugar using the DNS method (Miller, 1959). The concentration of reducing sugar was estimated from the D-galacturonic acid standard curve. The reaction mixture contained 400μL phosphate buffer (pH 7), 100 μL enzyme, and 500 μL pectin substrate. Control was run alongside experimental in which buffer was added instead of the enzyme. Test tubes were incubated at 30 ºC for 10 min. After incubation, the reaction was stopped by adding DNS and kept for 10 min at a boiling water bath. The absorbance of the reaction mixture was taken at 540nm using a spectrophotometer (Biz *et al*., 2014) (model, UV-1700, SHIMADZU Corporation, Japan) and the activity was calculated using the formula (1).

"One unit of polygalacturonase activity is defined as the amount of enzyme which is required to release the 1umol of galacturonic acid per minute under the standard assay conditions" (Khan *et al*., 2014).

2.5 Protein estimation

Bradford method was used for the estimation of total protein concentration using BSA as standard (Bradford, 1976).

$$\text{Enzyme unit (U/mL)} = \frac{\text{Concentration from graph}\left(\frac{mg}{ml}\right) \times \text{Dilution factor} \times 1000}{\text{Molecular weight of galacturonic acid} \times \text{Time of incubation}} \quad (1)$$

2.6 Molecular weight determination

The expression of polygalacturonase was determined by SDS-PAGE analysis (Mini-PROTEAN tetra Cell) (Laemmli, 1970; Manns, 2011).

2.7 Characterization of enzyme

2.7.1 Effect of temperature on enzyme activity and stability

At different temperatures i.e. 25 ºC-40 ºC, enzyme assay of the purified enzyme was carried out to analyze the effect of temperature on the enzyme activity. The thermal stability of polygalacturonase was checked by incubating the enzyme for 1-8h at various temperatures (20, 30, 40, 50, 60, and 70 ºC). After incubation at the optimum temperature and time, an activity assay was performed.

2.7.2 Thermodynamic parameters

The thermodynamic parameters such as activation energy (E_a), change in enthalpy (ΔH), and change in entropy (ΔS) were also evaluated by the Arrhenius plot (Anand *et al*., 2016).

2.7.3 Effect of pH on enzyme activity and stability

Sodium acetate buffer (pH 3.0-5.0) and phosphate buffer (pH 6.0-7.0) were used to study the effect of pH on the purified enzyme activity. At different time intervals (1-6h) by using buffers of different pH, the stability of the enzyme was studied under optimized conditions.

2.7.4 Effect of metal ions on enzyme activity

Enzyme activity was checked in presence of metal ions (Mn^{2+}, Co^{2+}, Mg^{2+}, Fe^{2+}, Zn^{2+}, Ca^{2+}, Hg^{2+}, Pb^{2+}, Ni^{2+}, Cd^{2+} and Cu^{2+}). The purified enzyme was incubated for 2h in the presence of metal ions solutions (2.5-10mM) and activity was performed under standard assay conditions afterward (Anand *et al*., 2016).

2.7.5 Effect of organic solvents on enzyme activity

The effect of organic solvents (acetone, toluene, methanol, ethanol, and DEE) on purified polygalacturonase was observed by incubating it in the presence of 2-10% organic solvents (Rehman *et al*., 2015).

2.7.6 Effect of inhibitors on enzyme activity

Different inhibitors/surfactants such as beta-mercaptoethanol, sodium dodecyl sulfate, tween-80, L-cysteine, and ethylene diamine tetra-acetic acid were used in different concentrations varying between 0.5-2.5mM to check their effect on enzyme activity. After incubation, the influence of various surfactants on enzyme activity was studied (Anand *et al*., 2017).

2.7.7 Substrate specificity

The effect of substrate specificity on enzyme activity was observed by using 1% citrus pectin, xylan, galactose, apple pectin, and Carboxymethyl cellulose (CMC) (Oumer and Abate, 2018).

2.7.8 Kinetic studies

The influence of various concentrations of a substrate on enzyme activity was studied using Line Weaver-Burk's (1934) double reciprocal Plot, and kinetic parameters "Km" and "Vmax" were determined.

2.8 Polygalacturonase immobilization

2.8.1 Calcium alginate beads

To immobilize the enzyme, sodium alginate (2%)and polygalacturonase were mixed in a 1:1 ratio and was then added dropwise in (0.2M) $CaCl_2$ solution, which leads to the formation of calcium alginate beads (Darah *et al*., 2015). The beads formed were washed with 0.1M sodium acetate buffer (pH) to eliminate the entrapped enzyme molecules. The calcium alginate beads were stored at 4ºC for further analysis.

2.8.2 Nanoemulsions

Dextran solution (2%) and enzyme were mixed in a ratio of 1:4 with continuous stirring for 10 minutes. Later polyethylene glycol (PEG) solution (1.6%) was added dropwise in it. The solution was frozen at -20ºC for 24h. followed by freeze-drying for the preparation of solid particles loaded with pectinase (Yuan *et al*., 2009). Polygalacturonase-loaded dextran particles were dissolved in 5ml of whey protein. After that 24mL of whey protein (2.5%) and 1mL sunflower oil were added to it. This mixture was then homogenized by bath sonication for 15 minutes. Then ultra-sonication was carried out at 60% amplitude, 30-sec pulse rate. Particle size was measured afterward by the zeta particle size analyzer (Kumar and Kumar, 2018).

2.8.3 *The shelf life of the free and immobilized enzyme*

The shelf life of both the free and immobilized enzymes was analyzed at various temperatures i.e. -20 ºC, 4 ºC, and 25 ºC. Activity assay was carried out daily until enzyme activity was diminished (De Lima *et al*., 2018).

2.8.4 *Clarification of fresh apple juice*

The effect of purified polygalacturonase on the clarification of fresh apple juice was observed. In the control tube distilled water was taken and the experimental tube contained purified enzyme. At a ratio of (1:4) in experimental and control tubes, fresh juice was added and incubated for 4h. at 30ºC and 120rpm. The absorbance and reducing sugar content of the reaction mixture were recorded at 520nm every hour. The difference in absorbance and reducing sugar content over time signifies the degree of clarification. (Uzuner, 2018; Cardoso*et al*., 2019).

2.8.5 *Statistical analysis*

All the experiments proceeded in triplicates. The computer statistical software Costat, cs6204W.exe was utilized for the statistical analysis of the results. A significant difference among the replicates has been presented as Duncan's multiple range tests in the form of probability (p) values (Snedecor and Cochran, 1980).

2.8.6 *Materials*

All the chemicals and material used in this research work were purchased from authentic companies such as Sigma, Fischer scientist, Merck, and Acros Ltd.

3 Results and discussion

3.1 *Polygalacturonase production and optimization of carbon sources*

The production of polygalacturonase was observed for 9 days. The enzyme activity and total protein content increased initially and reached their maximum value of (80.5 ± 0.03U/mL) and (0.96mg/mL ± 0.01), respectively on the 5th day of incubation and then started to decrease afterward as shown in figure 1A. Among different substrates, wheat bran enhanced the enzyme production as a carbon source followed by orange peel, apple peel, and lemon peel. Whereas rice straw inhibited the enzyme production as shown in figure 1B.

The polygalacturonase yield increase gradually and maximum enzyme production was attainted on the 5th day of incubation and decreased afterward possible due to decline in microbial growth, depletion of nutrients, and production of secondary metabolites (Cardoso*et al*., 2019). Anand *et al*. (2017) and Ahmed *et al*. (2019) reported similar findings from *A. niger*, while Doughari and Oynebarachi (2019) reported maximum polygalacturonase production from *A. flavus* on the 4th day of incubation which is contradictory to our findings probably because of different fungal strain and fermentation medium.

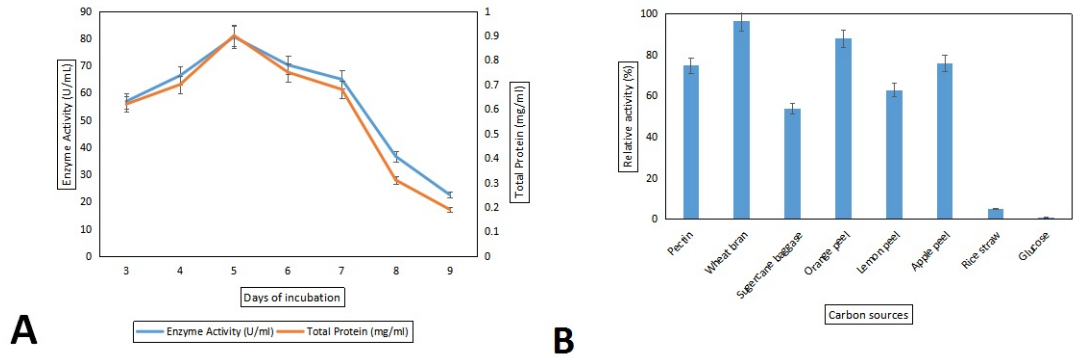

Fig. 1. (A) Effect of incubation period on enzyme and protein production, (B) Effect of different carbon source on polygalacturonase production. Y-error bars indicate the standard deviation (±SD), which differs significantly at $p \leq 0.05$.

The yield of polygalacturonase varies with different carbon sources because of the difference in amylase to amylopectin quantity present in substrates. Maller *et al*. (2011) reported Orange peel as the best carbon source for polygalacturonase production. Contrarily, Jahan *et al*. (2017) reported wheat bran as the best Carbon source for polygalacturonase production while Pagnoncelli *et al*. (2019) stated passion fruit peel as the best carbon source for polygalacturonase production by *P. janthinellum*. According to the reported results, the type of substrate present in the culture media affects the production of different isoenzyme.

3.2 Purification

The enzyme was precipitated between 30-80% fractions, and specific activity of 133.21±0.04 U/mg was observed. After dialysis, the concentrated protein was loaded to anion exchange column equilibrated with 50mM Tris-Cl buffer. After IEX, two peaks were obtained as shown in figure 2A. Activity assay was done for both peaks, the first shows 80% activity as compared to the second one. So, purified polygalacturonase was present in peak 1 which was further confirmed by SDS-PAGE. The purification fold of 2.98 was achieved whereas specific activity was enhanced to 256±0.04 U/mg. The percentage yield was calculated as 75%. The molecular weight was analyzed as 38 kDa with a single band on gel showing the purity of enzyme as shown in figure 2B. The overall summary of polygalacturonase purification is given in table 1.

About 2.98 folds' purification was obtained after ion-exchange chromatography with a specific activity of 256U/mg. Ma *et al*. (2016) used the DEAE-Sepharose FF column to elute purified protein with a molecular weight (38kDa) similar to our findings. Amin *et al*. (2017) reported the 3.07 folds' purification and two isoforms of *P. notatum* having molecular weights of 85 and 20kDa. Mahesh *et al*. (2016) purified polygalacturonase from *A. ibericus* and obtained 10 folds' purification using DEAE cellulose with molecular weight 41 kDa. Jurick *et al*. (2010) reported 41kDa molecular weight of PG obtained from *P. expansum*. Munir *et al*. (2020) obtained 2.27-fold purification and 51.25% yield of polygalacturonase from *A. tamarii*. The contrary results may be due to differences in variability of strain and different methods adopted for purification.

3.3 Enzyme characterization

3.3.1 Effect of temperature on enzyme activity and stability

Maximum activity (104.03 ± 0.04U/mL) was attained at 50ºC. The reduced enzyme activity (70.2± 0.05 U/mL) was noted at 60ºC as shown in figure 3A. The stability of polygalacturonase was checked by incubating an enzyme for 8h in a temperature range between 20-70ºC.

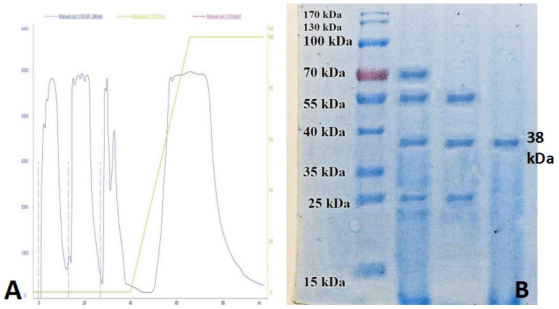

Fig. 2. (A) Purification of Polygalacturonase using Hitrap QFF using Bio-Rad UnosphereTMQ (BioscaleTM mini) column. (B) SDS-PAGE analysis of the purified polygalacturonase; Lane 1: Protein marker (Fermentas SN 0431), Lane 2: Crude Pectinase, Lane 3: Precipitated Pectinase, Lane 4: Purified Pectinase (38 kDa).

Table 1. Overall summary of Polygalacturonase purification.

Sample	Total units (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude enzyme	80500	960	83.85	100	1
Ammonium sulphate precipitation	75000	563	133.21	87	1.58
Anion exchange chromatography	60500	236	256	75	2.98

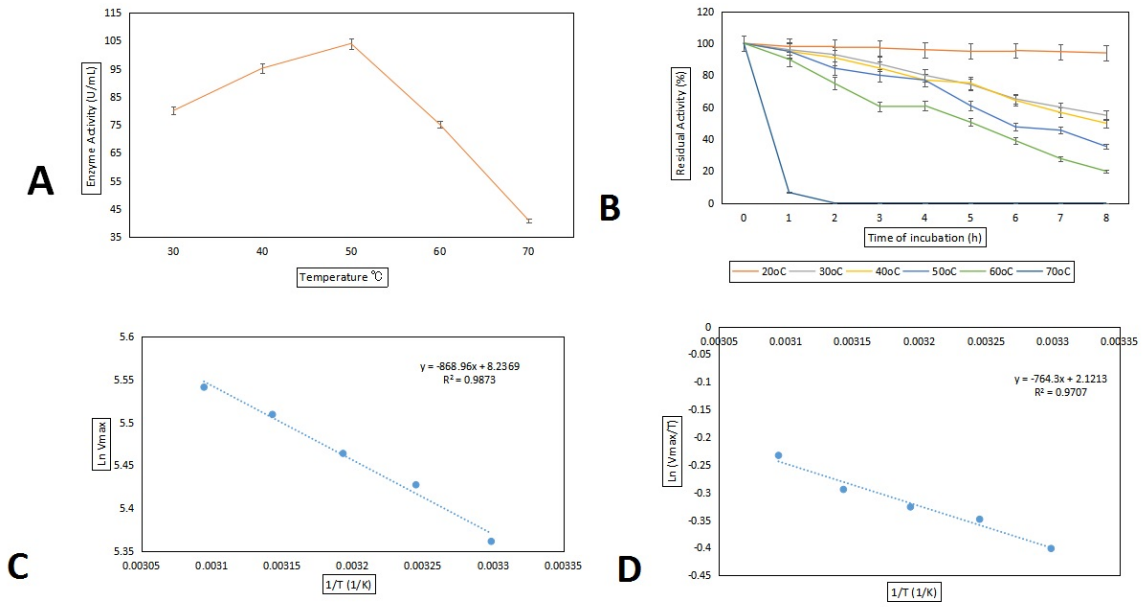

Fig. 3. Effect of different (A) temperatures, (B) incubation time on purified polygalacturonase. Arrhenius plot to calculate (C) the activation energy (E_a) and (D) the enthalpy of activation (ΔH) of purified polygalacturonase. Y-error bars indicate the standard deviation (±SD), which differs significantly at $p \leq 0.05$.

As illustrated in figure 3B, there was no change in residual activity initially, but it started to decline afterward. After 8 h. minimum residual activity of 50% was observed when incubated at 50 ºC. The enzyme retained its residual activity of 55% and 20% after 4 and 5 hours when incubated at 40 ºC and 60 ºC, respectively. At 70 ºC, the drastic decrease in the stability occurred i.e. 6.5% residual activity after 1hr which further declined and reached nil after 6 h. of incubation. This behavior is possibly due to the effect of heat on weak interactions of protein which resulted in the denaturation of the enzyme.

The optimum temperature for purified polygalacturonase was 50ºC. The result showed that polygalacturonase retained its activity at high temperatures. The optimum temperature of polygalacturonase is consistent with the results reported by Dey and Banerjee, 2014; Amin *et al*. 2017; Anand *et al*. 2017 who worked on *P. janthinellum*, *A. niger*, and *A. awamori*,respectively. On the other hand, the optimum temperature of polygalacturonase from *A. niger* was reported to be 37ºC by Gomes *et al*. (2011) and 40 ºC by Egwim and Caleb (2015) which is contrary to our result. Yadav *et al*. (2012) and Anand *et al*. (2017) reported 50ºC as optimum temperature for polygalacturonase obtained from *Rhizopus oryzae* and *A. niger*. Munir *et al*. (2020) mentioned 50ºC as optimum temperature for polygalacturonase obtained from *A. tamarii*. Aisien and Ikenebomeh, (2017) stated that the enzyme lost its activity at 70ºC due to partial denaturation which is quite similar to our results as, after 1hr, the enzyme lost its activity at 70ºC. Ire and Vinking (2016) reported that theenzyme from *A. niger* was 100% stable at 20ºC which is in accordance with our results. Almulaiky *et al*. (2020) detected PG obtained from *A. niger* is stable up to 50ºC which relates to our results.

3.3.2 Thermodynamic analysis

For thermodynamic analysis, the Arrhenius plot (Fig 3C and 3D) was considered to determine the activation energy (E_a), enthalpy of activation (ΔH), and entropy of activation (ΔS) which were found as 6.35 KJ/mol, 3.67 KJ/mol, and -1.1KJ/mol, respectively.

From the Arrhenius plot, thermodynamics parameters such as activation energy (E_a), enthalpy of activation (ΔH), and entropy of activation (ΔS) were found as 6.35 KJ/mol, 3.67 KJ/mol, and -1.1KJ/mol, respectively. Low values of enthalpy show the resistant nature of the enzyme (Ortega *et*

al.,2004). Khan *et al*. (2017) reported comparable results to the present study i.e. positive value of activation energy (26.3 KJ/mol) and negative value of entropy (-1.48KJ/mol). Amin *et al*. (2017) stated activation energy and enthalpy change of exo-polygalacturonase from *P. notatum* as 51.66, 44.06KJ/mol, and 41.39KJ/mol. These low values suggest that a stable bond was formed between the enzyme and substrate. Whereas, the low value of enthalpy change shows that an active complex is formed between enzyme and substrate (Ortego *et al*.,2004).

3.3.3 Effect of pH on enzyme activity and stability

A periodic increase in enzyme activity occurred up to pH 5. The maximum enzyme activity (105± 0.03 U/mL) was seen at pH 5. Afterward, activity started to decline as shown in figure 4A. The enzyme was incubated with buffers having a pH range between 3.0 to 7.0 for 6 h. At pH 5, 84% stability was obtained after 6 h of incubation. Whereas, it showed stability of up to 69%- 71% at pH 3.0-4.0, respectively for 6 h. The decrease in residual activity was more robust at pH 6.0 and 7.0 i.e. 20% and 9.5%, respectively, as shown in figure 4B.

The optimum pH of the enzyme was 5, and at pH 3.0-4.0, it was 84% and 71% stable. According to Kant's (2013) classification, pectinase is usually considered as acidic pectinase and can be used for the clarification of juices and wines. Ahmed *et al*. (2016) reported that pectinase obtained from Aspergillus niger was most active at pH 5, and similarly, Ma *et al*. (2016) also stated pH 5 as optimum pH for enzyme activity from *P. janthinellum*. Pagnonceli *et al*. (2019) reported that polygalaturonase obtained from *P. janthinellum* is 80% stable between pH 3-5 which is in accordance with our results while Torimiro *et al*. (2018) depicted that pH varies between 4-8. Zaslona and Trusek-Holownia (2015) reported maximum activity at pH 5,and 57% and 85% stability at pH 3.0 and 4.0, respectively which is close to our results.

3.3.4 Effect of metal ions on enzyme activity

No significant increase in enzyme activity was observed by the addition of metal ions (Mn^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Ca^{2+}, and Cu^{2+}), and K^+ and Na^+ slightly enhanced the enzyme activity as shown in figure 5. The activity of the enzyme decreased with the increase in the concentration of metal ions. Mercury (Hg^{2+}) had a negative effect on enzyme activity at all concentrations. Cd^{2+} and Pb^{2+} also acted as inhibitors with increasing concentrations. The pattern of enzyme activity was illustrated in figure 5A. Polygalacturonase activity was enhanced by K^+ and inhibited by Hg^+. As all metal ions used were heavy metals which can induce allosteric changes in the catalytic site of the enzyme. Enhancement of enzyme activity by potassium and sodium was presented by Nazir *et al*. (2019). Anand *et al*. (2016) reported a slight increase in enzyme activity by K^+ and Cu^{2+}. Potassium increases the enzyme activity as it stabilizes the native structure of the enzyme (Graza *et al*., 2013). Aisien and Ikenebomeh (2017) discussed the increase in polygalacturonase activity with the increase in metal ions concentration by suggesting that discrepancy of metal ions have a different effect on the flexibility of the active site of the enzyme. Yu and Xu (2018) stated that Mn^{2+} inhibited enzyme activity.

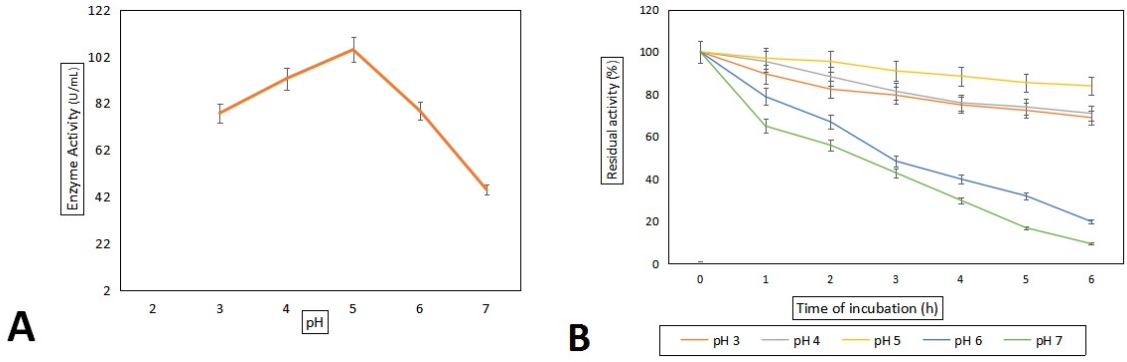

Fig. 4. (A) Effect of different pH on purified polygalacturonase activity by *P. notatum* MAIIB-33, (B) Effect of pH on stability of purified polygalacturonase by *P. notatum* MAIIB-33. Y-error bars indicate the standard deviation (±SD), which differs significantly at $p \leq 0.05$.

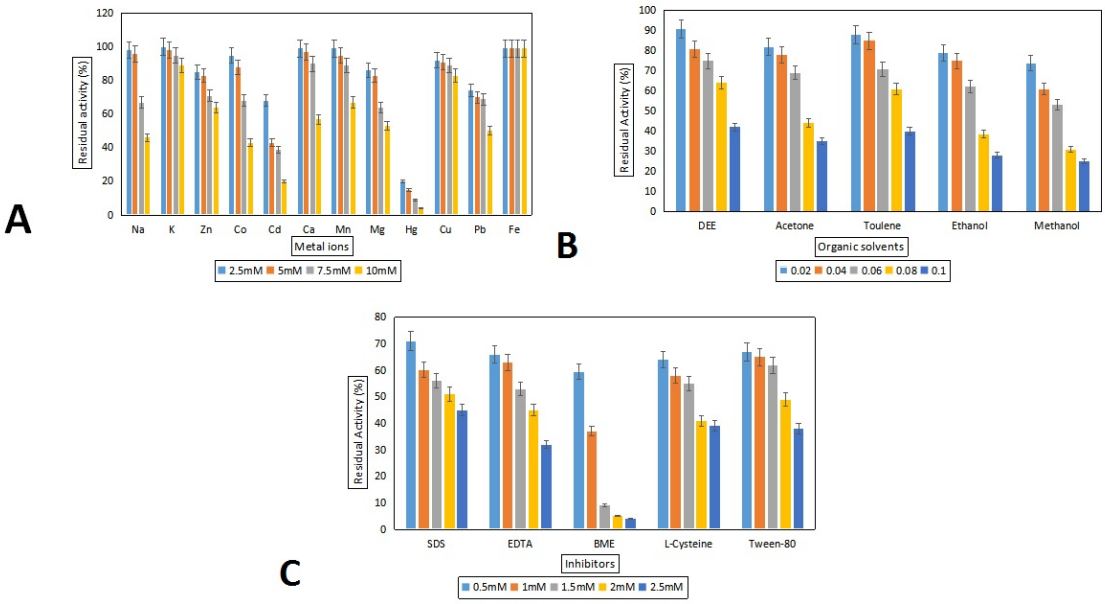

Fig. 5. Effect of different concentrations of (A) metal ions(2.5mM-10mM), (B) organic solvents (2-10%), (C) inhibitors/ surfactants (0.5-2.5mM) on purified polygalacturonase activity by *P. notatum* MAIIB-33. Y-error bars indicate the standard deviation (±SD), which differs significantly at $p \leq 0.05$.

3.3.5 Effect of organic solvents on enzyme activity

The enzyme activity decreased with an increase in the concentration of organic solvents. It was most notable for acetone, methanol, and ethanol with minimum residual activity obtained as at 35%, 25%, and 28%, respectively as compared to inorganic solvents such as Diethyl-ether (DEE) and toluene for which minimum enzyme activity was 42% and 40%, respectively. For organic solvents, similar results were presented by Okonji *et al*. (2019) for polar and non-polar organic solvents which are used in our study as the decrease was more drastic for organic solvents as compared to non-organic solvents. Pectin is insoluble in non-polar organic solvents (Toulene, DEE). Due to this reason in the presence of non-polar organic solvents concentration of pectin in an aqueous solution is higher (Okonji *et al*., 2019).

3.3.6 Effect of inhibitors on enzyme activity

After incubating an enzyme with different concentrations of inhibitors it was revealed that a minimum residual activity of 4% was exhibited in the presence of Beta-mercaptoethanol (BME). However, it retained activity of up to 45%, 32%, 39%, and 38% in the case of 2.5mM SDS, EDTA, L-cysteine, and Tween-80, respectively (figure 5C).

The reason for the decrease in activity by inhibitors is that all the inhibitors used are chelating agents, which inhibit the enzyme activity. Oumer and Abate (2018) stated that inhibitors like EDTA, Tween 20, and 80 enhanced with relative activity of enzyme from *Bacillus subtilis* while according to Okonji *et al*. (2019) at higher concentrations of EDTA, L-cysteine, SDS and Tween-80 inhibited the enzyme activity from *A. fumigatus*.

3.3.7 Substrate specificity

By incubating the enzyme with different substrates at optimum pH and temperature, its substrate specificity was determined. The highest activity was observed with citrus pectin whereas, little activity was observed with xylan and no activity with CMC. These results show that enzymes prefer hydrolyzing low esterified Pectins (Martins *et al*., 2013). The relative activity of polygalacturonase with the different substrates is shown in figure 6A.

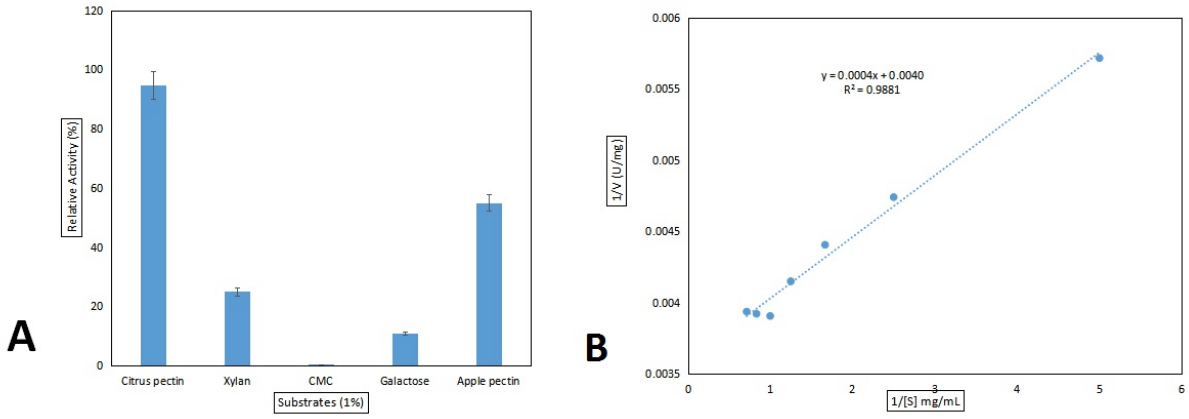

Fig. 6. (A) Effect of substrate specificity on polygalacturonase activity by *P. notatum* MAIIB-33, (B) Line Weaver-Burke Double Reciprocal Plot to calculate the "Km" and "Vmax" for purified polygalacturonase by MAIIB-33. Y-error bars indicate the standard deviation (±SD), which differs significantly at $p \leq 0.05$.

The most suitable substrate for polygalacturonase activity was found to be citrus pectin. Chowdhury *et al*. (2017) also reported the best activity with pectin and no activity with CMC. Cheng *et al*. (2016) reported no activity of polygalacturonase with xylan and CMC. Martin *et al*. (2013) stated citrus pectin as the best substrate for polygalacturonase produced by *Thermoascus auranticus*.

3.3.8 Kinetic analysis

Kinetic parameters i.e. "Km" and "Vmax" were calculated by using the Lineweaver-Burk double reciprocal plot. The "Vmax" was calculated as 250 U/mg while "Km" was 0.11 mg/ml for polygalacturonase as shown in figure 6B. Meena *et al*. (2015) found the value of "Km" for pectinase synthesized from Aspergillus spp. to be 2.43mg/ml. Amin *et al*. (2017) calculated "Vmax" and "Km" as 20 μmol/mL/min and 16.6mg/ml for pectinase produced from *P. notatum*. The maximum value of "Km" and "Vmax" reported by Laha *et al*. (2014) reported was 1mg/mL and 78U, respectively. Manal *et al*. (2016) reported "Km" and "Vmax" from *Paenibacillus lactis* to be 0.772 and 7.936, respectively. The low value of "Km"shows that the substrate has a high affinity for the enzyme. From the present study, it can be concluded that pectin can be used as a specific substrate for polygalacturonase industrially because the value of "Km" is not only comparable but is also in agreement to report of Rombouts and Pilnik (1980) according to which, "Km" of fungal pectinases is less than 1mg/mL.

3.4 Comparison of shelf life of the free and immobilized enzyme

Storage stability of enzyme was studied at different temperatures i.e. -20 ºC, 4 ºCand 25 ºC (figure 7A). The free enzyme was 58.2% stable for 15 days at -20 ºC, and 7 days at 4 ºC. The drastic decrease in enzyme activity was observed at 25 ºC having a shelf life of 3 days. Figure 7B depicts that the enzyme immobilized by alginate beads remained 74% stable for 15 days at -20 ºC. Whereas at 4 ºC and 25 ºC, the immobilized enzyme was found to be stable for up to 9 and 5 days, respectively. Enzyme immobilized by Nanoemulsions showed 80.2% stability at -20 ºC after 15 days (figure 7C). At 4 ºC, the gradual decrease in activity occurred every day till the 11th day and then reached zero. Nano-emulsions remained stable for 8 days at 25 ºC with little decrease in the activity of the immobilized enzyme.

It was concluded that the enzyme immobilized by Nanoemulsions was more stable at -20 ºC, 4 ºC, and 25 ºC in comparison with the free and immobilized enzyme by calcium alginate beads. The more stabilization effect is due to the protective microenvironment provided by the Nanoemulsions. Thus, it prevents the deformation of the active site of the enzyme which may be caused by an aqueous medium. Oliveria *et al*. (2018) stated that at 4 ºC immobilized enzyme from *A. aculeatus* was 32.3% stable after 12 days, Li *et al*. (2018) reported that immobilized pectinase has higher storage stability than free enzyme.

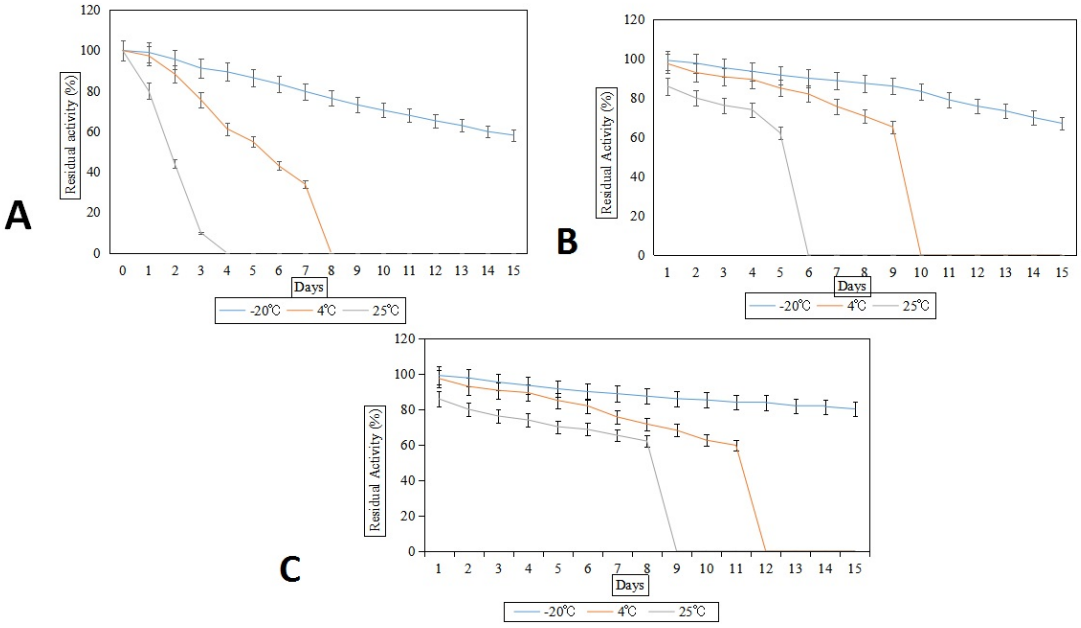

Fig. 7. Comparison of shelf life of (A) free, (B) immobilized enzyme by alginate beads and (C) nano emulsions (C). Y-error bars indicate the standard deviation (±SD), which differs significantly at $p \leq 0.05$.

3.5 Application in fruit juice clarification

The purified enzyme was used to test its potential in the clarification of apple juice by incubating it with the commercial enzyme for 4h. Commercial polygalacturonase was used as control. The experimental enzyme showed more clarification of juice in the experimental tube in comparison with control. With the clarification of fruit juice, the amount of reducing sugar increased more in experimental enzyme tube as compared to commercial enzyme. Moreover,turbidity and absorbance decreased as illustrated in figure 8A and B. This shows that polyalacturoannse purified and characterized in this research has potential for industrial use.

Polygalacturonase clarified the apple juice well because the pH of apple juice ranges from 3.3-4. So, there is close proximity between the pH of the juice and the pH stability of the enzyme. This results in greater clarification and increased catalytic activity of enzymes (Anand *et al*.,2017). KC *et al*. (2020) stated that the commercial potential of polygalacturonase can be exploited by yield, cost of enzyme production, and stability. Khan and Aruna (2017) and Nazir *et al*. (2019) reported similar results for clarification of apple pulp. Roy *et al*. (2018) also reported similar results for apple juice clarification. Amin *et al*. (2017) reported a reduction in apple juice turbidity using polygalacturonase produced by *P. notatum* which is in similarity to our work.

Conclusions

Penicillium notatum was reported to be a potent source of polygalacturonase production using agro-industrial waste, which can reduce production costs. As it is stable in acidic pH, the enzyme was discovered to be an excellent agent for clarifying fresh apple juice. *P. notatum* is widely used for the production of extracellular enzymes. The polygalacturonase activity was greatly affected by various agents, which resulted in a purification fold of 2.98. The enzyme's shelf life was significantly extended through the use of immobilisation techniques, making its use on an industrial scale more cost-effective. The storage stability, low cost, reusability, and inertness of this immobilised enzyme will prove beneficial for industrial applications. In the future, this purified enzyme can be subjected to protein engineering to enhance its temperature and pH stability.

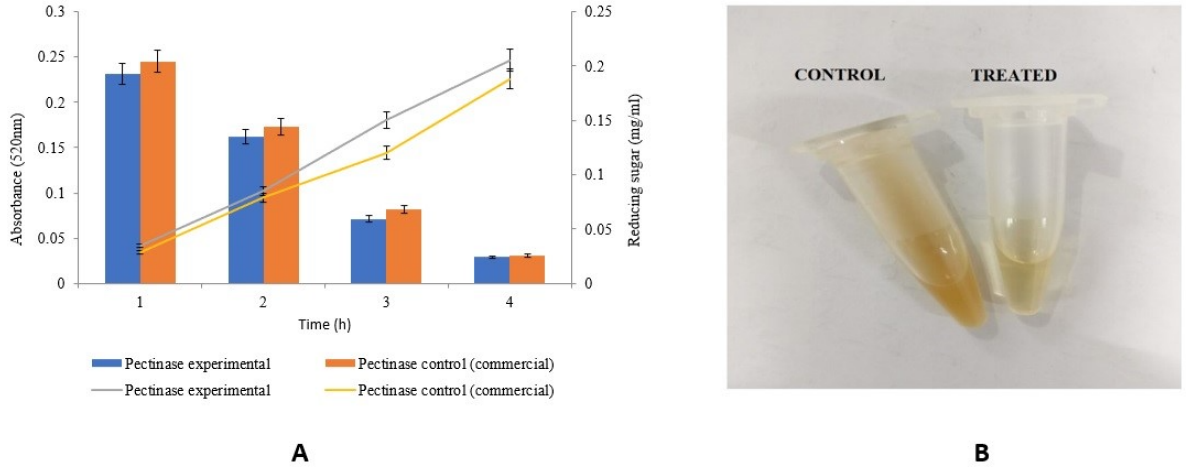

Fig. 8. (A) Effect of enzyme on optical density and reducing sugar content of fresh apple juice. Y-error bars indicate the standard deviation (±SD), which differs significantly at $p \leq 0.05$. (B) Clarification of apple juice from purified polygalacturonase; Left: control; Right: experimental.

References

Ahmed, I., Zia, M.A., Hussain, M.A., Akram, Z., Naveed, M.T. and Nowrouzi, A. (2016). Bioprocessing of citrus waste peel for induced pectinase production by Aspergillus niger; its purification and characterization. *Journal of Radiation Research and Applied Sciences 9*, 148-154. https://doi.org/10.1016/j.jrras.2015.11.003.

Ahmed, S.A., Saleh, S.A., Abdel-Hameed, S.A. and Fayad, A.M. (2019). Catalytic, kinetic and thermodynamic properties of free and immobilized caseinase on mica glass-ceramics. *Heliyon 5*, e01674. https://doi.org/10.1016/j.heliyon.2019.e01674.

Aisien, E.T. and Ikenebomeh, M.J. (2017). Production, Purification and comparison of polygalacturonase from Aspergillus flavus, Aspergillus niger and Penicillium expansum. *African Scientist 18*, 85-98. http://www.klobexjournals.com.

Almulaiky, Y., Albishri, A.A., Khalil, N.M., Algamal, Y., Aldhahri, M., Al-Harbi, S.A., Al-Talhi, H.A. and Alhadi, R. (2020). Polygalacturonase by Aspergillus Niger using seaweed waste under submerged fermentation: production, purification and characterization. *Biomedical Journal of Scientific and Technical Research 25*, 19416-19422. https://biomedres.us/pdfs/BJSTR.MS.ID.004249.pdf

Amin, F., Bhatti, H.N., Bilal, M. and Asgher, M. (2017). Purification, kinetic, and thermodynamic characteristics of an exo-polygalacturonase from *Penicillium notatum* with industrial perspective. *Applied Biochemistry and Biotechnology 183*, 426-443. https://doi.org/10.1007/s12010-017-2455-y.

Anand, G., Yadav, S. and 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*, 1-7. https://doi.org/10.1007/s13205-016-0517-4.

Anand, G., Yadav, S. and Yadav, D. (2017). Production, purification and biochemical characterization of an exo-polygalacturonase from Aspergillus niger MTCC 478 suitable for clarification of orange juice. *3 Biotech 7*, 1-8.https://doi.org/10.1007/s13205-017-0760-3.

Arogunjo, A.O. and Arotupin, D.J. (2018). Millet

cobs: a source of microbial enzymes. *Journal of Microbiology Biochemical Technology 10*, 124-133. 10.4172/1948-5948.1000405.

Bharadwaj, P.S. and Udupa, P.M. (2019). Isolation, purification and characterization of pectinase enzyme from Streptomyces thermocarboxydus. *Journal of Clinical Microbiology Biochemical Technology 5*, 001-006.http://dx.doi.org/10.17352/jcmbt.000031.

Bhardwaj, V., Degrassi, G. and Bhardwaj, R.K. (2017). Microbial pectinases and their applications in industries: A review. *Polymer 4*. https://www.irjet.net/archives/V4/i8/IRJET-V4I8144.pdf

Biz, A., Farias, F.C., Motter, F.A., De Paula, D.H., Richard, P., Krieger, N. and Mitchell, D.A. (2014). Pectinase activity determination: an early deceleration in the release of reducing sugars throws a spanner in the works. *PLoS One 9*, e109529. https://doi.org/10.1371/journal.pone.0109529.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry 72*, 248-254. https://doi.org/10.1016/0003-2697(76)90527-3.

Cardoso, F.D.S.N., Koblitz, M.G.B., Ortiz, G.M.D., Carvalho, J.L.V.D. and Carvalho, L.M.J.D. (2019). Study of the parameters used in the encapsulation of commercial pectinase in calcium alginate and its effect on its catalytic activity. *Food Science and Technology 39*, 247-252. https://doi.org/10.1590/fst.31518.

Cheng, Z., Chen, D., Lu, B., Wei, Y., Xian, L., Li, Y. and 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*, 989-998. https://doi.org/10.4014/jmb.1511.11045.

Chowdhury, T.I., Jubayer, M.F., Uddin, M.B. and Aziz, M.G. (2017). Production and characterization of pectinase enzyme from *Rhizopus oryzae*. *Potravinarstvo Slovak Journal of Food Sciences 11*, 641-651.https://doi.org/10.5219/656.

Dange, V.U., and Harke, S. (2018). Production and purification of pectinase by fungal strain in solid-state fermentation using agro-industrial bioproduct. *International Journal of Life Science Research 6*, 85-93.

Darah, I., Nisha, M. and Lim, S.H. (2015). Polygalacturonase production by calcium alginate immobilized Enterobacter aerogenes NBO2 cells. *Applied Biochemistry and Biotechnology 175*, 2629-2636. https://doi.org/10.1007/s12010-014-1447-4.

De Lima, J.S., Cabrera, M.P., De Souza, M.C.M., Converti, A. and Carvalho Jr, L.B. (2018). Hydrolysis of tannins by tannase immobilized onto magnetic diatomaceous earth nanoparticles coated with polyaniline. *Food Research International 107*, 470-476. https://doi.org/10.1016/j.foodres.2018.02.066.

De Oliveira, R.L., Dias, J.L., Da Silva, O.S. and Porto, T.S. (2018). Immobilization of pectinase from Aspergillus aculeatus in alginate beads and clarification of apple and umbu juices in a packed bed reactor. *Food and Bioproducts Processing 109*, 9-18. https://doi.org/10.1016/j.fbp.2018.02.005.

Dey, T.B. and Banerjee, R. (2014). Application of decolourized and partially purified polygalacturonase and α-amylase in apple juice clarification. *Brazilian Journal of Microbiology 45*, 97-104. https://doi.org/10.1590/S1517-83822014000100014.

Doughari, J.H. and Onyebarachi, G.C. (2019). Production, purification and characterization of polygalacturonase from Aspergillus flavus grown on orange peel. *Applied Microbiology: Open Access 4*, 1-7. https://doi.org/10.4172/2471-9315.1000155.

Egwim, E.C. and Caleb, T. (2015). Production and partial characterization of pectinase from snail (*Archachatina marginata*) gut isolates. *Biotechnology Journal International 5*, 196-205. https://doi.org/10.9734/BBJ/2015/11449.

Garza-Ramos, G., Mújica-Jiménez, C. and Muñoz-Clares, R.A. (2013). Potassium and ionic

strength effects on the conformational and thermal stability of two aldehyde dehydrogenases reveal structural and functional roles of K^+-binding sites. *PLoS One 8*, e54899. https://doi.org/10.1371/journal.pone.0054899.

Gomes, J., Zeni, J., Cence, K., Toniazzo, G., Treichel, H. and Valduga, E. (2011). Evaluation of production and characterization of polygalacturonase by Aspergillus niger ATCC 9642. *Food and Bioproducts Processing 89*, 281-287. https://doi.org/10.1016/j.fbp.2010.10.002.

Ire, F.S. and Vinking, E.G. (2016) Production, purification and characterization of polygalacturonase from Aspergillus niger in solid state and submerged fermentation using banana peels. *Journal of Advance Biology and Biotechnology 10*, 1-15. http://dx.doi.org/10.9734/JABB/2016/29593

Jahan, N., Shahid, F., Aman, A., Mujahid, T.Y. and Qader, S.A.U. (2017). Utilization of agro waste pectin for the production of industrially important polygalacturonase. Heliyon, 3(6), e00330. https://doi.org/10.1016/j.heliyon.2017.e00330.

Javed, R., Nawaz, A., Munir, M., Hanif, M., Mukhtar, H., Haq, I.U. and Abdullah, R. (2018). Extraction, purification and industrial applications of pectinase: A review. *Journal of Biotechnolology and Bioresearch 1*, 1-6. https://doi.org/10.31031/JBB.2018.01.000503.

Jurick, W.M., Vico, I., Gaskins, V.L., Garrett, W.M., Whitaker, B.D., Janisiewicz, W.J. and Conway, W.S. (2010). Purification and biochemical characterization of polygalacturonase produced by *Penicillium expansum* during postharvest decay of 'Anjou' pear. *Phytopathology 100*, 42-48. https://doi.org/10.1094/PHYTO-100-1-0042.

Kant, S., Vohra, A. and Gupta, R. (2013). Purification and physicochemical properties of polygalacturonase from Aspergillus niger MTCC 3323. Protein Expression and Purification, 87(1), 11-16.https://doi.org/10.1016/j.pep.2012.09.014.

Sundeep, K.C., Upadhyaya, J., Joshi, D.R., Lekhak, B., Kumar, C.D., Raj, P.B. and Raghavan, V. (2020). Production, characterization, and industrial application of pectinase enzyme isolated from fungal strains. Fermentation, 6(2), 59. https://doi.org/10.3390/fermentation6020059

Khadija, T.K., Sikander, A. and Hira, S.M. (2018) Potential applications of pectinases in food, agricultural and environmental sectors. *Journal of Pharmacy Chemical Biological Science 6*, 23-34.

Khan, N.J., Ali, N. and Hamid, M. (2017). Kinetic and thermodynamic analysis of purified pectinases produced from nitrous acid mutant derivative of Aspergillus niger (H-97). *Pakistan Journal of Botany 49*, 2321-2324.

Khan, S., Saim, S. and Abid, A. (2014) Isolation and selection of Aspergillus species for hyper-production of polygalacturonases. *Pakistan journal of Biochemical Molecular Biology 47*, 125-128.

Kumar, H. and Kumar, V. (2018). Ultrasonication assisted formation and stability of water-in-oil nanoemulsions: optimization and ternary diagram analysis. *Ultrasonics Sonochemistry 49*, 79-88. https://doi.org/10.1016/j.ultsonch.2018.07.022.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature 227*, 680-685. https://doi.org/10.1038/227680a0.

Laha, S., Sarkar, D. and Chaki, S. (2014). Optimization of production and molecular characterization of pectinase enzyme produced from Penicillium chrysogenum. *Scholars Academic Journal of Biosciences 2*, 326-335. ISSN 2321-6883.

Li, Y., Liu, J., Zhang, K., Lei, L. and Lei, Z. (2018). UiO-66-NH2 PMAA: A hybrid Polymer-MOFs architecture for pectinase immobilization. *Industrial and Engineering Chemistry Research 57*, 559-567. https://doi.org/10.1021/acs.iecr.7b03398.

Lineweaver, H. and Burk, D. (1934). The determination of enzyme dissociation constants. *Journal of the American Chemical Society*

56, 658-666. https://doi.org/10.1021/ja01318a036.

Ma, Y., Sun, S., Hao, H. and Xu, C. (2016). Production, purification and characterization of an exo-polygalacturonase from Penicillium janthinellum sw09. Anais da Academia Brasileira de Ciências, 88, 479-487. https://doi.org/10.1590/0001-3765201620150051.

Mahesh, M., Arivizhivendhan, K.V., Maharaja, P., Boopathy, R., Hamsavathani, V. and Sekaran, G. (2016). Production, purification and immobilization of pectinase from Aspergillus ibericus onto functionalized nanoporous activated carbon (FNAC) and its application on treatment of pectin containing wastewater. *Journal of Molecular Catalysis B: Enzymatic 133*, 43-54. https://doi.org/10.1016/j.molcatb.2016.07.012.

Maller, A., Damásio, A.R.L., Silva, T.M.D., Jorge, J.A., Terenzi, H F. and Polizeli, M.D.L.T.D. M. (2011). Biotechnological potential of agro-industrial wastes as a carbon source to thermostable polygalacturonase production in *Aspergillus niveus*. *Enzyme Research 2011*, 6. https://doi.org/10.4061/2011/289206.

Manal, S.S., Sahar, S.M., Manal, G.M., Mohsen, M.A. and Osama, H.E. (2016). Purification and kinetics of pectinase production from Paenibacillus lactis NRC1 locally isolated from egyptian mangrove habitat. *DER Pharma Chemica 8*, 150-159.

Manns, J. M. (2011). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins. *Current Protocols in Microbiology 22*, A-3M. https://doi.org/10.1002/9780471729259.mca03ms22.

Martins, E.D.S., Leite, R.S.R., Da Silva, R. and Gomes, E. (2013). Purification and properties of polygalacturonase produced by thermophilic fungus Thermoascus aurantiacus CBMAI-756 on solid-state fermentation. *Enzyme Research 2013*, 7. https://doi.org/10.1155/2013/438645.

Meena, K.K., Jaipal, M.K. and Singh, U. (2015). Production kinetics and characterization of pectinase enzyme from *Aspergillus niger*. *South Asian Journal of Food Technology and Environment 1*, 131-135.

Miller, G.L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical chemistry, 31(3), 426-428. https://doi.org/10.1021/ac60147a030.

Mohandas, A., Raveendran, S., Parameswaran, B., Abraham, A., Athira, R.S., Mathew, A.K. and Pandey, A. (2018). Production of pectinase from Bacillus sonorensis MPTD1. *Food Technology Biotechology 56*, 110. https://doi.org/10.17113/ftb.56.01.18.5477.

Munir, M., Abdullah, R., Haq, I., Kaleem, A., Iqtedar, M. and Ashraf, S. (2020). Purification, characterization, kinetics and thermodynamic analysis of polygalacturonase from Aspergillus tamarii for industrial applications. *Revista Mexicana de Ingeniería Química 19*, 293-304. https://doi.org/10.24275/rmiq/Bio1753.

Nazir, Z., Ijaz, S., Gul, R. and Saleem, M. (2019). Purification and characterization of an acidic polygalacturonase from grapes and its potential to improve juice quality. *Pakistan Journal of Zoology 51*, 1387-1402. http://dx.doi.org/10.17582/journal.pjz/2019.51.4.1387.1402.

Obafemi, Y.D., Ajayi, A.A., Taiwo, O.S., Olorunsola, S.J. and Isibor, P. O. (2019). Isolation of polygalacturonase-producing bacterial strain from tomatoes (Lycopersicon esculentum Mill.). *International Journal of Microbiology 2019*, 9. https://doi.org/10.1155/2019/7505606.

Okonji, R.E., Itakorode, B.O., Ovumedia, J.O. and Adedeji, O.S. (2019). Purification and biochemical characterization of pectinase produced by *Aspergillus fumigatus* isolated from soil of decomposing plant materials. *Journal of Applied Biotechnology 7*, 1-8. http://dx.doi.org/10.7324/JABB.2019.70301.

Ortega, N., De Diego, S., Perez-Mateos, M. and Busto, M.D. (2004). Kinetic properties and thermal behaviour of polygalacturonase used in fruit juice clarification. *Food Chemistry 88*, 209-217. https://doi.org/10.1016/j.foodchem.2004.01.035.

Oumer, O.J. and Abate, D. (2018). Comparative studies of pectinase production by Bacillus subtilis strain Btk 27 in submerged and solid-state fermentations. *BioMed Research International 2018*, 10. https://doi.org/10.1155/2018/1514795.

Pagnonceli, J., Rasbold, L.M., Rocha, G.B., Silva, J.L.C., Kadowaki, M.K., Simão, R.D.C.G. and Maller, A. (2019). Biotechnological potential of an exo-polygalacturonase of the new strain *Penicillium janthinellum* VI2R3M: biochemical characterization and clarification of fruit juices. *Journal of Applied Microbiology 127*, 1706-1715. https://doi.org/10.1111/jam.14426.

Patidar, M.K., Nighojkar, S., Kumar, A., and Nighojkar, A. (2018). Pectinolytic enzymes-solid state fermentation, assay methods and applications in fruit juice industries: A review. *3 Biotech 8*, 1-24. https://doi.org/10.1007/s13205-018-1220-4.

Rebello, S., Anju, M., Aneesh, E.M., Sindhu, R., Binod, P. and Pandey, A. (2017). Recent advancements in the production and application of microbial pectinases: An overview. *Reviews in Environmental Science and Biotechnology 16*, 381-394. https://doi.org/10.1007/s11157-017-9437-y.

Rehman, H.U., Aman, A., Nawaz, M.A. and Qader, S.A.U. (2015). Characterization of pectin degrading polygalacturonase produced by Bacillus licheniformis KIBGE-IB21. *Food Hydrocolloids 43*, 819-824.https://doi.org/10.1016/j.foodhyd.2014.08.018.

Rombouts, F.M. and Pilnik, W. (1980). Pectic enzymes. In *Economic Microbiology, Vol. 5: Microbial Enzymes and Bioconversions* (pp. 227-282). Academic Press.

Roy, K., Dey, S., Uddin, M., Barua, R. and Hossain, M. (2018). Extracellular pectinase from a novel bacterium Chryseobacterium indologenes strain SD and its application in fruit juice clarification. Enzyme Research, 2018, 7. https://doi.org/10.1155/2018/3859752.

Sandri, I.G. and Silveira, M.M.D. (2018). Production and application of pectinases from Aspergillus niger obtained in solid state cultivation. Beverages, 4(3), 48. https://doi.org/10.3390/beverages4030048.

Snedecor, G.W. and Cochran, W.G. (1980). *Statistical Methods* Iowa State University Press, Ames. Statistical methods, 7th ed. The Iowa State University Press, Ames.

Siddiqui, M., Pande, V. and 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

Singh, J., Kundu, D., Das, M. and Banerjee, R. (2019). Enzymatic processing of juice from fruits/vegetables: An emerging trend and cutting edge research in food biotechnology. In *Enzymes in Food Biotechnology* (pp. 419-432). Academic Press. https://doi.org/10.1016/B978-0-12-813280-7.00024-4

Torimiro, N., Adediwura, V.A., Ojo, S.T., Oluwadare, A.O. and Okonji, R.E. (2018). Pectinolytic activities of pectinase produced by some bacterial isolates cultured from deteriorating fruits. *Nigerian Journal of Biotechnology 35*, 91-98. https://doi.org/10.4314/njb.v35i2.12.

Ubani C.S., Ezugwual, A.L., Obinna, A.O., Santos, G.A.M. and Ekwedigwe, O.A. (2015) Isolation, partial purification and characterization of pectinase from water melon (*Citrullus lanatus*) rind. *American-Eurasian Journal of Toxicological Sciences 7*, 110-114.

Usha, D.K., Kanimozhi, G. and Panneerselvam, A. (2014). Isolation and screening of pectin lyase producing fungi from soil sample of dead organic matters. *World Journal of Pharmaceutical Research 3*, 563-569.

Uzuner, S. (2018). Effect of crude pectinase on apple juice quality characteristics by desirability approach. *Gida 43*, 558-568. https://doi.org/10.15237/gida.GD18024.

Yadav, S., Anand, G., Dubey, A.K. and Yadav, D. (2012). Purification and characterization of an exo-polygalacturonase secreted by *Rhizopus oryzae* MTCC 1987 and its role in retting of *Crotalaria juncea* fibre. *Biologia 67*, 1069-1074. https://doi.org/10.2478/s11756-012-0122-x.

Yu, P. and Xu, C. (2018). Production optimization, purification and characterization of a heat-tolerant acidic pectinase from *Bacillus* sp. ZJ1407. *International Journal of Biological Macromolecules 108*, 972-980. https://doi.org/10.1016/j.ijbiomac.2017.11.012.

Yuan, W., Geng, Y., Wu, F., Liu, Y., Guo, M., Zhao, H. and Jin, T. (2009). Preparation of polysaccharide glassy microparticles with stabilization of proteins. *International Journal of Pharmaceutics 366*, 154-159. https://doi.org/10.1016/j.ijpharm.2008.09.007

Zaslona, H. and Trusek-Holownia, A. (2015). Enhanced production of polygalacturonase in solid-state fermentation: selection of the process conditions, isolation and partial characterization of the enzyme. *Acta Biochimica Polonica 62*. http://dx.doi.org/10.18388/abp.2015_1081.