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Purification, characterization, kinetics and thermodynamic analysis of polygalacturonase from *Aspergillus tamarii* for industrial applications

Análisis de purificación, caracterización, cinética y termodinámica de poligalacturonasa de *Aspergillus tamarii* para aplicaciones industriales

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Abstract

Increasing industrial demand has urged the scientists to biosynthesize polygalacturonase (PG) that would be operative, stable, cost effective and can be used in industrial applications particularly in fruit juice clarification and poultry feed. In current work, polygalacturonase was produced by submerged fermentation employing *Aspergillus tamarii* strain SA-11 having Genbank accession number MG772816. The enzyme purification and characterization from *Aspergillus tamarii* was the main focus of the current research work. Fractionation by ammonium sulphate along with ion exchange chromatography was used for purification and 2.27 purification fold with 51.25% yield was attained. SDS-PAGE analysis showed the molecular mass of purified enzyme as 70kDa. Enzyme kinetic assessment i.e. K_m 2.85 mg/mL and V_{max} 55.55 in addition with thermodynamic determinants such as Ea=-39.84 KJ/mol, ΔH=37.62 KJ/mol and ΔS=-38.05 KJ/mol proves industrial stability of this enzyme. Further, different parameters were characterized which revealed that enzyme remained stable up to 50 ºC and pH 8. Furthermore, PG found to retain its activity in the presence of different metals whereas some inhibitors reduced its activity i.e. PMSF and EDTA. The enzyme was found to have a better shelf life of 30 days at -20 and 4 ºC compared to room temperature. The reduction in turbidity of fresh juice (40%) and increase in body weight of chicks (1550±1.30 g) feeding on feed treated with purified PG proves its efficiency in industrial applications. This study concluded PG as multi stress stable enzyme with appreciable potential to be employed in fruit juice clarification and poultry feed digestibility improvement.

Keywords: Polygalacturonase, catalysis, clarification, stability, thermodynamics, kinetics.

Resumen

La creciente demanda industrial ha instado a los científicos a biosintetizar la poligalacturonasa (PG) que sería operativa, estable, rentable y puede usarse en aplicaciones industriales, particularmente en la clarificación de jugos y la alimentación de aves de corral. En el trabajo actual, la poligalacturonasa se produjo por fermentación sumergida empleando la cepa *Aspergillus tamarii* con el número de registro de Genbank MG772816. La purificación y caracterización enzimática de *A. tamarii* fue el foco principal del trabajo de investigación actual.El fraccionamiento por sulfato de amonio junto con la cromatografía de intercambio iónico se utilizó para la purificación y se obtuvo un pliegue de purificación de 2,27 con un rendimiento de 51,25%. El análisis SDS-PAGE mostró la masa molecular de la enzima purificada como 70 kDa. La evaluación cinética de la enzima, es decir, K_m 2.85 mg/mL y V_{max} 55.55 además de determinantes termodinámicos como Ea = -39.84 KJ / mol, ΔH = 37.62 KJ / mol y ΔS = -38.05 KJ/mol demuestra la estabilidad industrial de esta enzima. Además, se caracterizaron diferentes parámetros que revelaron que la enzima permaneció estable hasta 50 ° C y pH 8. Además, PG encontró que retiene su actividad en presencia de diferentes metales, mientras que algunos inhibidores redujeron su actividad, es decir, PMSF y EDTA. Se encontró que la enzima tenía una mejor vida útil de 30 días a -20 y 4 ºC en comparación con la temperatura ambiente. La reducción en la turbidez del jugo fresco (40%) y el aumento en el peso corporal de los pollitos (1550±1.30 g) que se alimentan de alimento tratado con PG purificado demuestran su eficacia en aplicaciones industriales. La estabilidad de la poligalacturonasa purificada en condiciones ambientales adversas y sus aplicaciones exitosas en la industria alimentaria y avícola la convierten en una de las enzimas más exigentes para futuros propósitos.

Palabras clave: Poligalacturonasa, catálisis, clarificación, estabilidad, termodinámica, cinética.

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1 Introduction

Polygalacturonase (PG) is one of the most important enzymes that are responsible for cleaving pectin, a complex structure found in the cell wall of different plants that gives strength, hardness and firmness to them (Karahalil *et al*., 2017). This cleavage results in the formation of monogalacturonic acid due to which, the plant cell wall becomes softer and helps in obtaining high concentration of fruit juices with less pulp. Furthermore, breakage of plant cell wall makes plant based poultry feed more digestible for poultry chicks (Obafemi *et al*., 2019). Microbial production of this enzyme is preferred due to ease of synthesis and extraction. A variety of microorganisms including *Aspergillus niger, Aspergillus tamari, Aspergillus oryzae, Penicillium, Bacillus, Fusarium* and *Saccharomyces* species have been reported for its synthesis (Adedeji and Ezekiel, 2019).

The purification and characterization of the synthesized microbial enzyme are important steps that need to be carried out prior to its commercialization and industrial application as it helps in evaluating the molecular structure and kinetics of the enzyme and also provides an advantage in attaining maximal catalytic activity (Nazir *et al*., 2019).The extent to which the purity is desired is based on the utilization of the end product for specific purposes. For purifying enzymes, ammonium sulfate fractionation along with various chromatographic procedures such as ion exchange chromatography or size exclusion chromatography can be used (Castañeda-Casasola *et al*., 2018). Kinetic and thermodynamic evaluation is of great interest for determining the suitable substrate requirements of enzyme and plays a significant role with respect to economical view (Adingra *et al*., 2017). Similarly, enzyme characterization has gained importance as it aids in achieving highest yield of purified enzyme. If the enzyme has the ability to tolerate high temperature, pH conditions and is also resistant to metal ions presence, then that particular enzyme is considered feasible for use in harsh environmental conditions industries (Anand *et al*., 2016). Moreover, the shelf life of enzyme is a significant parameter that is based on the enzyme stability. The enzyme that remains active in wide temperature ranges for longer time period are usually considered as practicable and economical from industrial point of view (Tan *et al*., 2019). Therefore, purification and characterization of enzyme are great importance in fermentation industry.

Purified and characterized polygalacturonases can be employed commercially in a wide number of applications including mucilage separation from coffee beans, fermentation of tea, enhancing poultry digestion and attaining large amounts of clarified fruit juices (Fitch-Vargas *et al*., 2019). Oil extraction industries are also being using this enzyme from a decade to make the extraction process easy. All these applications have urged the researchers to produce purified and characterized polygalacturonases that would be cost effective and would have high efficiency, specificity and stability. Therefore, objective of the current study was to purify and characterize polygalacturonase produced from fungal specie *A. tamarii* for a number of industrial applications such as fruit juice clarification and preparation of more digestible poultry feed.

2 Materials and methods

2.1 Materials

Chemicals were purchased from Merck, Fischer scientific and Sigma Aldrich Ltd of microbiological analytical grades for use in current research.

2.2 Microorganism

We have previously reported the production of polygalacturonase by *A. tamarii* isolated from rotten fruit sample with GenBank accession number MG772816 (Munir, 2019). In present study, same microorganism was used for the production of polygalacturonase at pilot scale.

2.3 Production of polygalacturonase

2.3.1 Inoculum preparation

For preparation of spore inoculum, 100 mL autoclaved nutrient broth was inoculated with 1mL conidial suspension of *A. tamarii*, aseptically and allowed to incubate overnight at 30 ºC and 200 rpm.

2.3.2 Submerged fermentation

Fermentation experiment was carried out in 7.5L bioreactor (Model: BF-110 BioFlo/ CelliGen New Brunswick) using the fermentation medium (5L)

defined by Fratebianchi *et al*. (2017) with modification of using powdered form of cheeku coverings as Carbon source. The sterile fermentation medium was inoculated with 1% (v/v) of vegetative inoculum and placed for 3 days at temperature of 30 ºC and agitation rate of 200 rpm. The medium was kept at pH 6 with 1vvm aeration rate. After incubation, supernatant was separated from solid biomass by filtration through muslin cloth. The filtered supernatant was further centrifuged at 6000 rpm at 4 ºC (Avanti high speed centrifuge JX-26, Beckman Coulter, USA) to remove any of remaining solid debris. The supernatant obtained was used for the estimation of polygalacturonase (Abdullah *et al*., 2019).

2.4 Enzyme activity

The estimation of polygalacturonase activity was carried out by following the method of Okafor *et al*. (2010). One unit of PG was calculated as enzyme used for the production of 1 μg of galacturonic acid in one minute.

2.5 Purification

The crude enzyme was subjected to ammonium sulphate fractionation (10-60%) to remove the junk proteins (Saxena *et al*., 2003). After fractionation, excessive salt in partially purified fraction of PG was removed by membrane dialysis after Ma *et al*. (2016). Partially purified fraction of PG was subjected to anion exchange chromatography in which Bio-Rad Mini Macro-Prep diethyl amino ethyl (DEAE) column was loaded with 1 ml of the protein fraction (5 mg/mL) along with the binding buffer (0.1 M Phosphate buffer of pH 6) that helps in removing the unbound proteins as well as elution buffer (0.1 M Phosphate +NaCl buffer) that was needed to elute the bounded proteins at later stage after Marquez *et al*. (2017). Then purity and molecular mass of PG was determined by running the sample on SDS-PAGE (Pradal-Velázquez *et al*., 2018).

2.6 Enzyme kinetics and thermodynamic analysis

The specificity of PG to substrate and its stability under extreme temperature conditions was evaluated by analyzing its kinetic and thermodynamic parameters including K_m, V_{max}, activation energy, enthalpy change and entropy change using Line-weaver Burk plot and Arrhenius plots, respectively (Dalagnol *et al*., 2017).

2.7 Characterization of polygalacturonase

Influence of several physical and chemical factors such as influence of temperature, pH, metallic ions, surfactants, inhibitors and organic solvents on PG activity and stability were estimated. Furthermore, storage of purified PG was also deliberated by varying temperature conditions. Table 1 depicts the conditions at which all of these parameters were characterized (Jimenez-Rodriguez *et al*., 2020).

2.8 Industrial applications

2.8.1 Clarification of fruit juices

The clearing of cloudiness in apple fresh juice was assessed by incubating 10 mL of it with purified PG (0.5 mL) at 50 ºC for 30 min. The change in turbidity was determined by taking absorbance of the juice at a wavelength of 660nm before and after treatment along with change in reducing sugar content.

2.8.2 Enhancing poultry digestibility

For evaluating the influence of PG on poultry feed, 500 g feed was mixed with 100 mL of PG and incubated for about 6 h at 50 ºC. The treated feed was given to chicken in order to analyze its effect on their growth rate.

Table 1. Different parameters variables for characterization of purified PG.

Parameters	Variables
Temperature	20-60 ºC for activity and 20-80 ºC with 12 h incubation for stability.
pH	3-8 for activity and pH 3-8 with 12 h incubation for stability.
Metal ions	1mM Ca^{+2}, Cu^{+2}, Co^{+2}, Mg^{+2}, Mn^{+2}, Zn^{+2}, Cr^{+2}, Pb^{+2} and Hg^{+2}.
Surfactants	1% SDS, Tween 20, Tween 80, SDS, β-mercaptoethanol.
Inhibitors	1mM solutions of PMSF and EDTA.
Organic solvents	1% Ethanol, methanol, isopropanol, hexane and CCl_4
Shelf life	At 20 ºC, 4 ºC and -2 ºC for 30 days.

Four groups of chicken (each group have three chickens) were used for this purpose in which 3 flocks were served with feed dosed with PG whereas only one flock was fed on regular feed. Then after every week, chicks' weight was measured and the process was continued for about 6 weeks. At the end of the 6th week, relative growth rate was estimated by using the formula given below:

$$\text{RGR} = \frac{(\text{final weight-initial weight})}{[0.5 \times (\text{final weight-initial weight}) \times 100]} \quad (1)$$

2.9 *Statistical analysis*

All the experiments were conducted in triplicates and reliability of findings was evaluated through SPSS version 16.00. ANOVA was used for determining the alteration in probability values.

3 Results and discussion

3.1 *Biosynthesis of PG*

Considering the importance of incubation time for PG production, its synthesis was assessed for 12-84 h. A steady rise was detected in the PG synthesis from 12 to 48 h and highest production (52.01 U/mL/min) was attained at 60 h (Figure 1). Sethi *et al*. (2016) stated upmost synthesis of same enzyme after 96 h using *A. terreus*. The conflicting outcomes suggested that the enzyme synthesis and incubation interval differs according to the strain used. *A. terreus* may have slow metabolic growth rate compared to *A. tamarii* used in current study. Another reason might be the use of different fermentation medium which contributes largely towards synthesis rate of enzyme. In current study, maximum yield of PG with activity of 38.3±0.04 U/mL and 45.44±0.01 U/mL was achieved using wild and mutagenic strains of *A. tamarii* at agitation rate of 150 rpm which shows confliction with the study of Darah *et al*. (2013) and Ibrahim *et al*. (2015) that reported maximum PG biosynthesis at 200rpm using *A. niger*. The results of both the studies contradicts to the results of the present work that might be due to the high oxygen and continuous homogenous nutrients distribution requirements of *A. niger* as compared to *A. tamarii*.

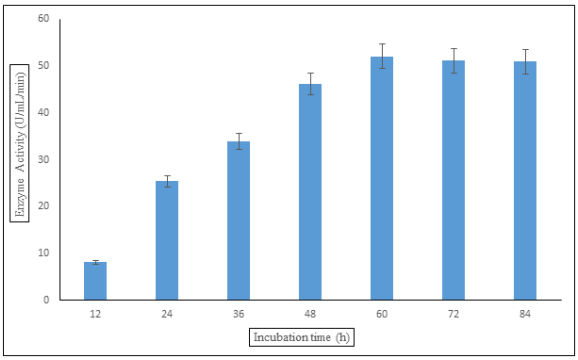

Fig. 1. Effect of incubation time on PG production using *A. tamarii* by submerged fermentation in a stirred bioreactor. Standard error was determined by calculating mean of triplicate results and represented by deviation bars.

3.2 *Purification*

3.2.1 *Ammonium sulfate fractionation*

As a first step of purification, ammonium sulphate fractionation was conducted to eliminate unwanted proteins and maximum PG was recovered in a fraction precipitated using 40% ammonium sulphate as evident from Figure 2a. Daniel *et al*. (2014) using *A. falvus* and Tan *et al*. (2020) employing *A. luchuensis* achieved maximum PG fractionation at 80% and 90% concentration of ammonium sulphate. Opposing verdicts can be associated to the alteration in PGs ionic strength biosynthesized by different isolates of *Aspergillus*. After fractionation excessive salt was removed from the partially purified fraction of PG by membrane dialysis using phosphate buffer (pH 6).

3.2.2 *Anion exchange chromatography*

Partially purified dialyzed fraction was subjected to ion exchange chromatography using anion exchanger DEAE column. The fraction collected for a peak obtained with elution buffer (0.1 M Phospahte +NaCl buffer) at 48 minutes of reaction time showed PG activity (Figure 2b). The purified fraction exhibited 57.57±0.05 U/mg specific activity with 2.27 purification fold and 61.25% purification yield (Table 2). Figure 2c depicts that the molecular mass was found to be 70 kDa employing SDS-PAGE. Similar results were reported by Daniel *et al*. (2014) who also used anion exchange chromatography as a method to achieve desired purification that indicates the presence of positive charge on the enzyme.

Table 2. Purification summary of PG produced by *A. tamarii*.

Sample	Total units (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude enzyme	161070	6360	25.32	100	1
Ammonium sulphate precipitation	122190	2940	41.56	75.86	1.64
Anion exchange chromatography	98670	1714	57.57	61.25	2.27

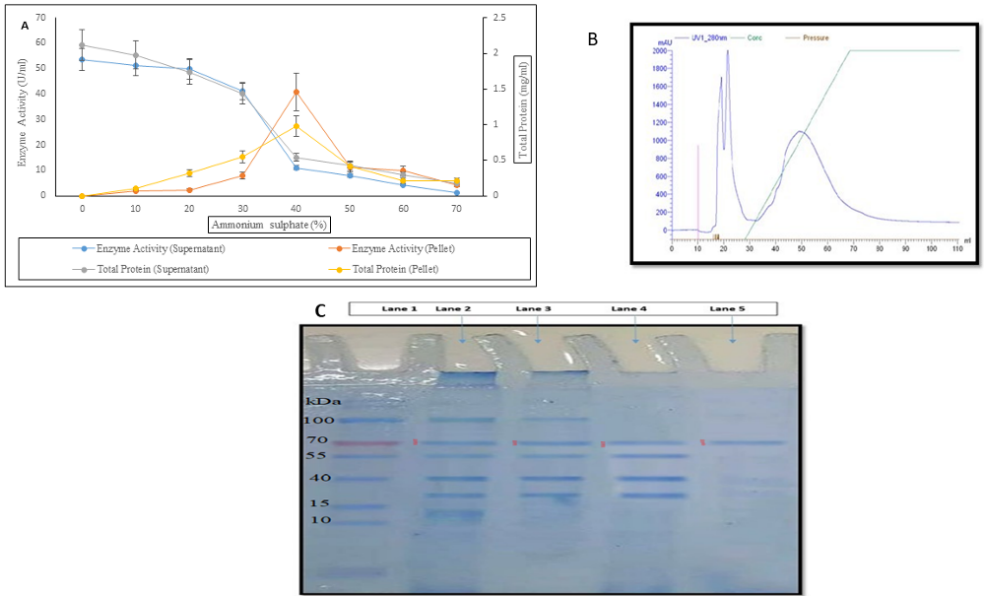

Fig. 2. (a) Ammonium sulphate fractionation profile of crude PG (b) Anion exhcnage chromatograph showing purification of PG (c) SDS PAGE gel for assessing the purity and molecular weight of purified PG (Lane 1: Protein Ladder, Lane 2: Crude enzyme, Lane 3: Ammonium sulphate precipitated fraction, Lane 4: Dialyzed Fraction. Lane 5: Ion exchange chromatography purified fraction).

The molecular mass estimated by Khatri *et al.* (2015) was 70 kDa that is in agreement to the current findings whereas Martos *et al.* (2014) found it to be 43kDa. The variations in results might be associated with the use of variant source for fungal isolation.

3.2.3 Kinetic and thermodynamic analysis

The specificity of polygalacturonase towards its substrate was determined by line weaver Burk plot method in which K_m and V_{max} were estimated as 55.55 U/mg and 2.85 mg/mL, respectively as indicated in figure 3a. On the other hand, thermodynamic parameters were accessed by Arrhenius plot. Using this plot, Ea, enthalpy and entropy change were determined as -39.84 KJ/mol, 37.62 KJ/mol and -38.05 KJ/mol, respectively as shown in figure 3b and 3c. The research work of Thakur *et al.* (2010) found the K_m and V_{max} as 0.22 mg/ml and 2.34 U/ml. The disagreement in results from present study was might be due to the use of alternative strain i.e. *R. pusillus*. The contradictory results show that the kinetic parameters of enzyme depend on the metabolic mechanism of the used microbial strain. As the strains used in both studies differ from each other, therefore, different kinetic analysis is obtained in both the cases. On the other hand, Trindade *et al.* (2016) reported activation energy of 125.5 kJ/mol and 50 ºCkJ/mol of enthalpy change.

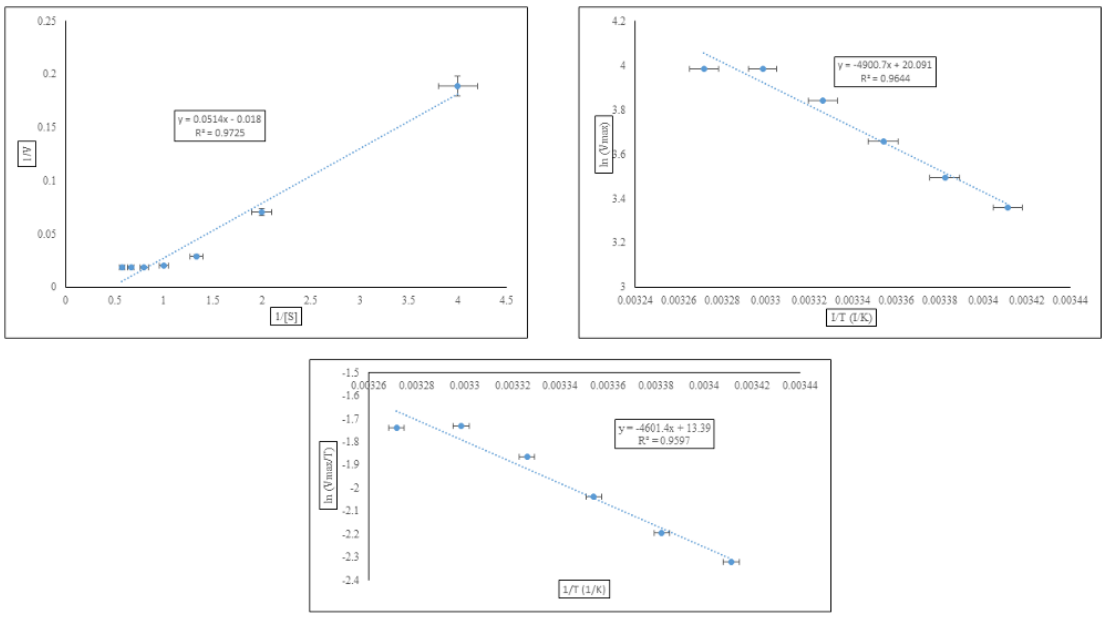

Fig. 3. Kinetic and thermodynamic analysis of purified PG: (a) Line weaver Burk plot for estimation of K_m and V_{max}, (b) Arrhenius plot for determination of Ea, (c) Arrhenius plot for estimation of enthalpy and entropy. Standard error was determined through calculating mean of triplicate results and represented by deviation bars.

The obtained results depict the improved and enhanced production of polygalacturonase. Therefore, it suggests that the enzyme purified in present work is more suitable for use under harsh environmental factors.

3.3 Polygalacturonase characterization

3.3.1 Temperature and pH impact on PG activity and stability

The incubation of PG at various temperatures and pH revealed that the maximum activity of 55.59±0.12 U/mg and 55.71±0.05 U/mg was obtained at 30 ºC (Figure 4a) and pH 5.5 (Figure 4c) which was remained constant up to 40 ºC and pH 6. Furthermore, the enzyme incubation for 12 hours showed that it retained its activity even at higher temperature (50 ºC) and pH 8 for up to 8 hours as evident from figures 4b and 4d, respectively. Giacobbe *et al*. (2014) performed his work on characterizing PG from *A. niger* that was also found to be stable at 50 ºC and 8.2 pH. Similarity in the findings was might be due to employing similar fungal genera. These results of both studies suggest that the polygalacturonase produced from *Aspergillus* species are thermo tolerant and alkaline tolerant in nature.

3.3.2 Metal ions and surfactants effect on enzyme activity and stability

In the presence of different metal ions, the highest yield was achieved with Ca^{+2} ions in the medium with relative activity of 110% and the enzyme remained stable for up to 12 hours. The relative activity and stability in the presence of all other metal ions is illustrated in figure 5a and 5b. Jagadeesh and Viswanathan (2010) reported similar findings in their study. However, Amid *et al*. (2012) cited that Ca^{+2} ions have negative impact on polygalacturonase activity. Among all the metal ions used mercury exhibited inhibitory effect on the activity of enzyme that corresponds to the study of Oumer and Abate (2017). These findings suggested us that mercuric ions have ability to bind with sulfhydryl groups of amino acid that brings overall damage to enzyme structure. Investigations to analyze the effect of surfactants (SDS, β-mercaptoethanol, Tween 20 and 80) on PG activity and stability revealed that β-mercaptoethanol have a worst effect on PG activity with residual activity of 67% (Figure 5c). Besides, all the surfactant showed negative effect on the stability of PG as can be observed in figure 5d.

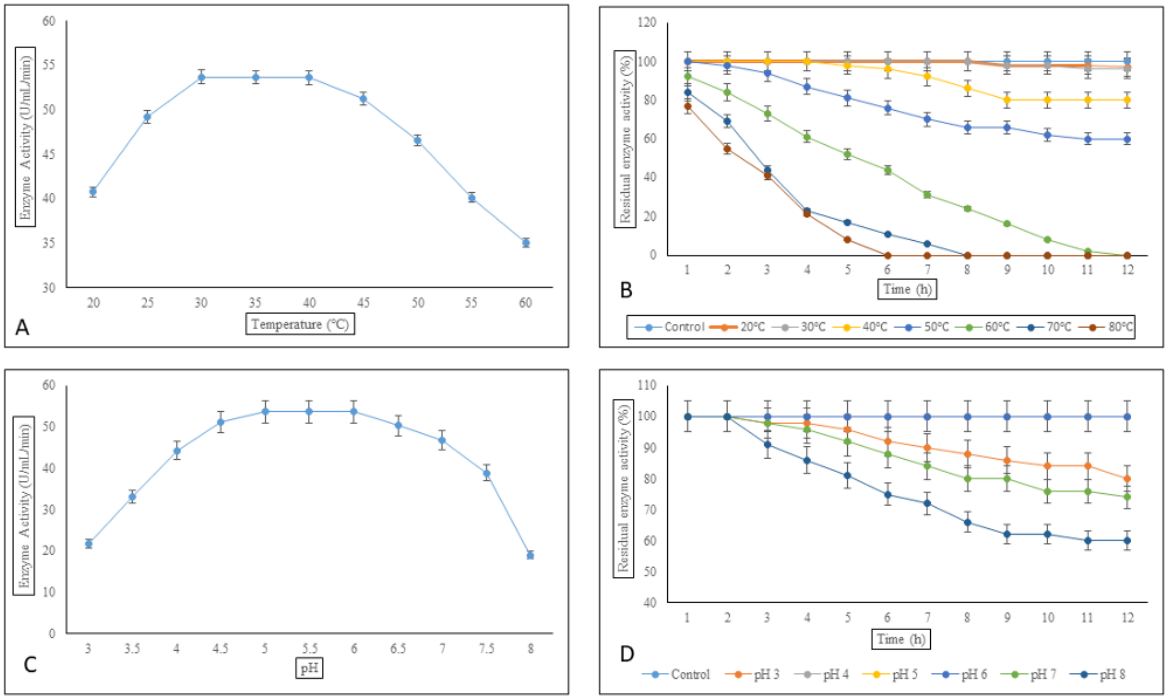

Fig. 4. Effect of temperature (4a, 4b) and pH (4c, 4d) on PG activity and stability. Standard error was determined by calculating mean of triplicate results and represented by deviation bars.

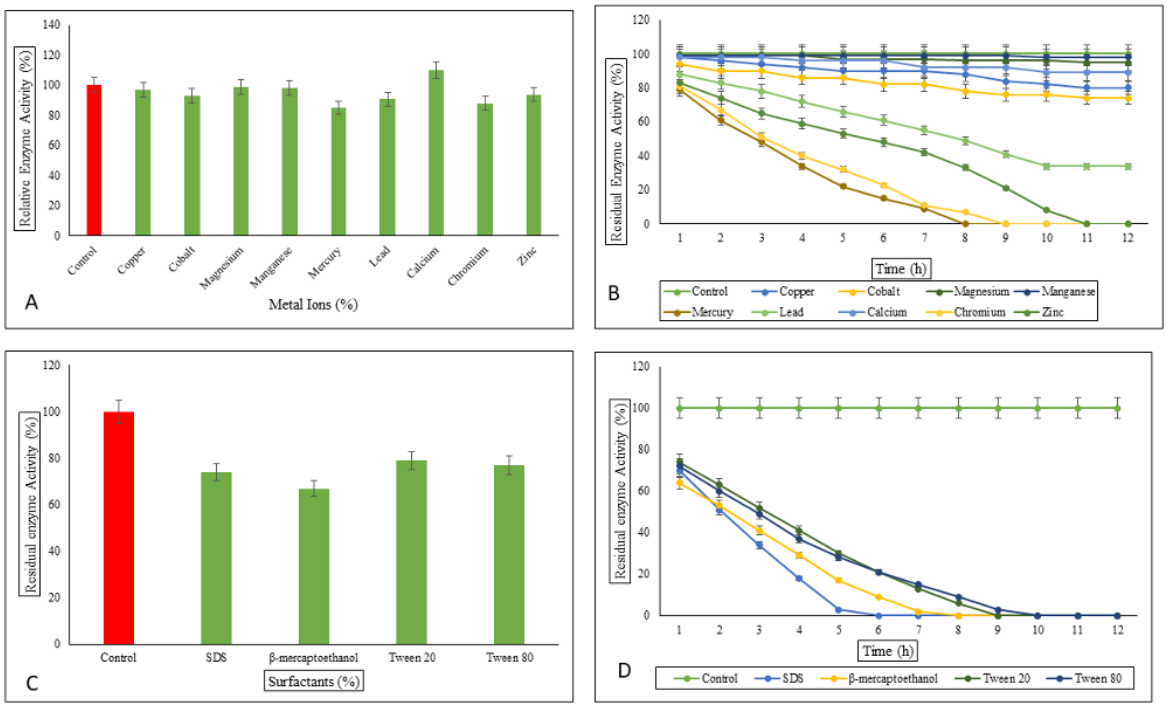

Fig. 5. Impact of metal ions (5a, 5b) and surfactants (5c, 5d) on PG activity and stability. Standard error was determined by calculating mean of triplicate results and represented by deviation bars.

Table 3. Effect of PG treated feed on weight gain (g) of poultry chicks.

Groups	Weight Day 1 (g)	Weight Week 1 (g)	Weight Week 2 (g)	Weight Week 3 (g)	Weight Week 4 (g)	Weight Week 5 (g)
Control (Poultry feed not treated with PG)	26 ±1.20	112 ±0.65	310 ±1.20	626± 1.70	1089 ±1.12	1499 ± 1.57
Experimental (Poultry feed treated with PG)	26 ±1.01	142 ±1.06	388 ±1.03	750±1.46	1207 ±0.98	1550 ± 1.30

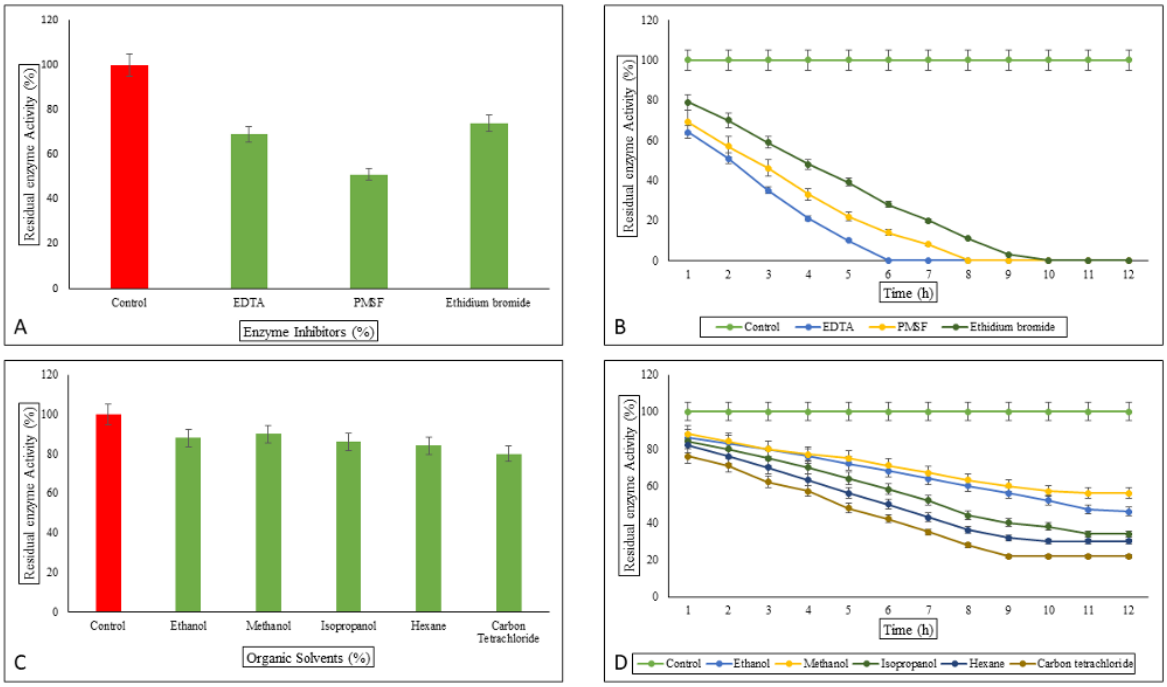

Fig. 6. Impact of enzyme inhibitors (6a, 6b) and organic solvents (6c, 6d) on PG activity and stability. Standard error was determined by calculating mean of triplicate results and represented by deviation bars.

3.4 Effects of inhibitors and organic solvents on activity and stability of PG

The incubation of PG with different inhibitors (PMSF, EDTA) and organic solvents (ethanol, methanol, tetra chloromethane) showed that tetrachloromethane and PMSF maximally reduced the activity of polygalacturonase to 80 and 51%, respectively (Figure 6a and 6c). A significant drop in the stability of enzyme was also observed after 3 h in the availability of all inhibitors and solvents (Figure 6b and 6d) which is similar to the finding presented by Li *et al*. (2012). The corresponding results suggest that PMSF and β-mercaptoethanol can cause enzyme inhibition by interacting with protein and changing the 3D enzyme structure.

3.5 Shelf life of polygalacturonase

The storage of polygalacturonase at varying temperatures for 30 days showed that it remained active at -20 and 4 ºC even after 30 days whereas a noticeable drop in its activity was observed at 25 ºC as shown in figure 7. Therefore, it is suggested that the enzyme can stay active at 4 and -20 ºC for about 30 days. Martin *et al*. (2019) also reported corresponding results that the enzyme can remain stable for 8 months after immobilization in hydrogel beads but the catalytic sites of enzymes can mix with substrate after longer durations. Another study of Deng *et al*. (2019) reports the opposite results that the immobilized enzyme remains stable and resistant for 60 days. The contradiction to the findings of current study can be associated to the use of free enzyme without any immobilization.

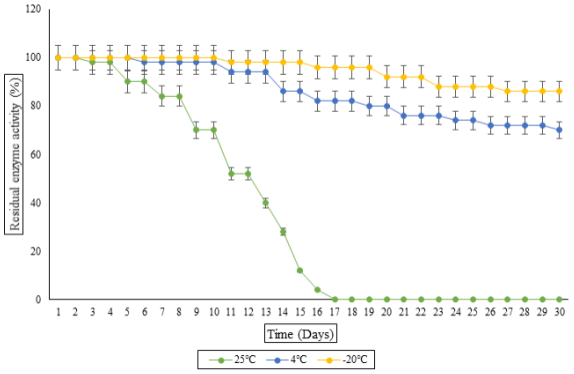

Fig. 7. Shelf life profile of purified PG at different storage temperatures. Standard error was determined by calculating mean of triplicate results and represented by deviation bars.

3.6 Industrial applications

3.6.1 Improved poultry feed digestibility

The purified polygalacturonase treated feed imparted a positive effect on the chicks body mass that was amplified excellently after 5th week i.e. 1550±1.30 g compared to chicks given untreated feed (Table 3). Comparable results were cited by Kutlu *et al.* (2019). These studies proposes that PG elevates the digestibility of chicks by removing non-starchy polysaccharides that have toxic effects and thus increases the feed quality that positively influences the poultry health.

3.6.2 Fruit juice clarification

Appreciable findings were observed in case of juice clarification in which purified PG reduces the turbidity of fresh apple juice by breaking down of pectic substances (Figure 8a). This resulted in increase of total reducing sugars content to 22.56 mg/mL compared to 9.56mg/mL in control (Figure 8b). Azar *et al.* (2020) also obtained similar results and reported the increases of reducing sugar concentration that is due to the polygalacturonase. The successful accomplishment of polygalacturonase in both the fields makes it realistic for use on commercial scale.

Conclusions

Purification and characterization of polygalacturonase have a profound effect on the industrial efficacy of enzyme. Anion exchange chromatography proved to be an efficient method for purification of PG with significant protein recovery. The factors like stability in wide pH and temperature range makes this enzyme suitable to work in harsh industrial conditions. Furthermore, Kinetic parameters endorsed the applicability of PG produced at industrial scale due to high catalytic activity. Significant impact of this enzyme on improvement of poultry feed digestibility and fruit juice clarification revealed it as an important enzyme with huge potential in market. However, with each passing day, the researchers should look for cheaper ways to produce economical polygalacturonase of good quality that would work more efficiently in food, poultry, fermentation and oil extraction industries.

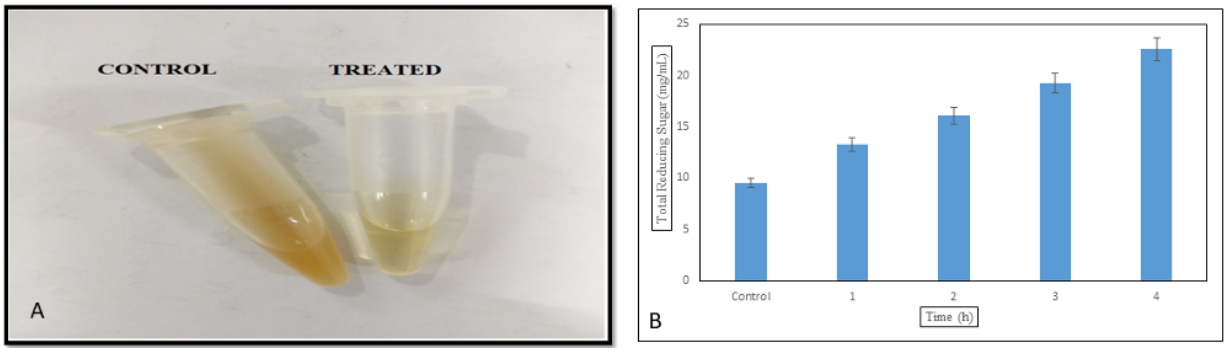

Fig. 8. Effect of purified PG on clarification (a) and total reducing sugar content (b) of fruit juice. Standard error was determined by calculating mean of triplicate results and represented by deviation bars.

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