Purification and Thermodynamic Characteristics of an Exo-Polygalacturonase from *Trichoderma Pseudokoningii*

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Summary: Polygalacturonases (PGs) are necessary to degrade the insoluble viscous pectin components during the clarification process of fruit juice and are produced by some plants and various microbes, such as bacteria, yeasts and fungi. In this study, an exo-polygalacturonase (exo-PGP4a) was purified from *T. Pseudokoningii* using DEAE-Sepharose and Sephacryl S-200 columns. We show that the enzyme produced in this study by solid-state fermentation of citrus Orange peel was purified 20-fold with 12.8% recovery. The apparent molecular mass of the enzyme was determined to be 25 kDa using gel filtration and SDS-PAGE. The optimum temperature and pH of the exo-PGP4a were 45°C and 6, respectively. The exo-PGP4a showed half-lives of 50.95 and 21.32 min at 55 and 75°C, respectively. The activation energy for denaturation (E_a^*) was 42.596 kJ/mol. The K_m value of the enzyme for PGA hydrolysis was 2 mg/ml, and the V_{max} was 3.27 µmol min^{-1} mg^{-1}. Several metal cations, such as $Cu^{2+}$and Zn^{2+}, were found to enhance the enzymatic activity of the exo-PGP4a, while Pb^{2+}, Ca^{2+}, Ni^{2+}, Cd^{2+}, Co^{2+} and Hg^{2+} ions were found to be inhibitory. In this study, we suggest the exo-polygalacturonase has potential role of the clarification of Orange, Apple, Grape, and Peach juices in the food industry.

Keywords: Exo-polygalacturonase, Purification, *T. Pseudokoningii,* Juice clarification.

Introduction

A pectinolytic enzyme has the potential to degrade pectin, which is found in the plant cell wall. This degradative enzyme is produced by various microorganisms, such as bacteria and yeast [1, 2]. A filamentous fungus is one of the most important sources to produce polygalacturonases (PGs) in industries and has a wide range of technical applications to produce simple sugar, bioethanol and oligosaccharides [3, 4]. PG is a widely studied pectinolytic enzyme [5]. This pectinolytic enzyme is produced by a fungus, which is why the World Health Organization (WHO) reported that fungi are an ideal source in food biotechnology [6]. Production using filamentous fungi is cost-effective compared with the extraction of whole cells, because they produce enzymes extracellularly. In industrial processes, the reduction in the time and cost of production is an important step. PG plays an important role in the juice industry by helping to remove the pectin fragments during processing, which not only saves the time of filtration but also improves the quality of the juices [7]. Although some bacterial PGs have been reported to have neutral and alkalin-optimal pH, most microbial polygalacturonases have optimum pH in the acidic range seven [8]. Optimal pH is an important point for determining the use of polygalacturonases in applications. The extraction and clarification of fruit juices were based on acidic polygalacturonases [9], whereas alkaline polygalacturonases are used in the recovery of natural fibres, the fermentation of coffee and tea leaves, the treatment of wastewater and the bioscouring of cotton [10]. Pectinases are divided into polygalacturonases, pectin lyases and pectin esterases according to their mode of action. Among the pectinolytic enzymes, polygalacturonases (exo and endo) are commonly and widely studied enzymes that catalyze the hydrolysis of α-1, 4 glycosidic bonds between two galacturonic residues and the pectin chain [11]. Also, pectin lyase finishes trans-elimination reactions and pectinesterase de-esterifies the pectin polymer following hydrolysis of ester linkages [12]. With regard to chemical techniques, the enzymatic degradation of pectin components is economically and environmentally achievable, as enzymes demonstrate elevated specificity, selectivity and activity under mild response circumstances [13]. Polygalacturonases are divided into exo- and endo-polygalacturonases on the basis of the enzyme classification. An exo-PG (E.C. 3.2.1.67) acts on the terminal monomer of polygalacyuronic acid and liberates monogalacturonic acid, while endo-PG (EC 3.2.1.15) acts on polygalacturonic acid and liberates oligogalacturonic acid [14]. The objective of this work was to investigate the production, purification and thermodynamic properties of exo-PG obtained from *T. Pseudokoningii* by solid-state fermentation (SSF) on citrus Orange peels.

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Experimental

Chemicals

Polygalacturonic acid (PGA) and monogalacturonic acid were obtained from Sigma Chemicals. Other chemicals for electrophoresis and column chromatography were obtained from Amersham Pharmacia Biotech.

Microorganisms

T. pseudokoningii was obtained from the Plant Pathology Unit, National Research Centre, Cairo, Egypt.

Cultivation of organisms

Five grams of citrus Orange peel were soaked with 5 mL distilled water in a 50 mL Erlenmeyer flask and autoclaved at 121°C pressure of 15 psi for 15 min. One ml of spore suspension of *T. pseudokoningii* was inoculated in the flask and incubated at 28°C for 7 days. Crude enzyme was extracted by centrifugation at 12000 rpm for 10 min and the supernatant was specified as a crude extract.

Purification of exo-polygalacturonase

The crude extract of exo-polygalacturonase was loaded into a DEAE-Sepharose column equilibrated with a buffer of 20 mM Tris-HCl, pH 7.2. The enzyme was eluted using a stepwise gradient (0.0 to 0.3 M NaCl). The eluents were collected (3 mL fractions each) and monitored at 280 nm for protein using a spectrophotometer (Jenway 6315 UV/Visible). Enzyme assays were performed for each protein fraction. The fractions exhibiting enzymatic activity were pooled (P1-P4). An exo-polygalacturonase (exo-PGP4) with the highest activity was loaded on a Sephacryl S-200 column. The eluents were collected (3 mL fractions each) at a flow rate of 30 mL / h.

Exo-polygalacturonase assay

Exo-polygalacturonase activity has been tested using PGA as the substrate [15]. Activity towards PGA was determined by the formation of reducing groups using the dinitrosalicylic acid reagent (DNS) [16]. The enzymatic reaction was performed in a 0.5 mL final volume. It contained 2% (w/v) PGA, 50 mM sodium acetate buffer, pH 5.5, and a suitable amount of enzyme. The assay was performed at 37°C for 1 h followed by the addition of DNS (0.5 mL). The reaction mixture was heated in a boiling water bath for 10 min followed by cooling to ambient temperature. The absorbance was measured at 560 nm.

Protein determination

Protein estimation was performed as previously described by Bradford [17], using BSA as a standard.

Protein molecular mass estimation

Gel filtration was used with Sephacryl S-200 to determine the protein's molecular mass. In addition, SDS-PAGE was used to determine the purification enzyme's purity and molecular mass subunit [18].

Mechanism of action of purified PGP4

To determine whether the purified PG is an endo- or exo-PG, five units of purified PG were added to 125 µL of PGA 2% (w/v), 125 µL of 0.2 M sodium acetate buffer pH 5.5, and 200 µL of distilled water. The reaction mixture was performed at 37°C for 30 min followed by cooling to ambient temperature. Thin layer chromatography was conducted using 2 µL of reaction solution. Spots of PGA and monogalacturonic acid were also added onto TLC, which was maintained in a container containing a solution of acetic acid, isopropanol and water (2: 5: 3, v/v/v) as the mobile phase. The chromatogram was air dried, sprayed with 10% sulphuric acid and 0.2% (w/v) orcinol in methanol and allowed to air dry. Chromatographic spots were later photographed.

Characterization of exo-polygalacturonase

pH optimum

Exo-PGP4 activity was detected at different pH values using different buffers, including 0.2 M sodium acetate (pH 4.0-6.0) and Tris-HCl (pH 6.5-9.0).

Temperature optimum

Exo-PGP4 activity was detected at various temperatures ranging from 30-55°C to determine the optimal temperature. Data were plotted after setting the maximum activity as 100%.

Thermodynamic study

Heating the purified enzyme in a wide range of temperatures of 55-75 °C was a test of the thermal stability profile, and calculating the remaining activity using the following equation from sterile aliquots removed at regular intervals:

Residual exo-PG activity (%) = C_t / C_0

where C_t and C_0 describe the activities at time t (min) and time t = 0 min.

The enthalpy (ΔH*) was calculated using the relationship given in the following equation

$\Delta H^* = E_a^* - RT$

where R = 8.314 J K^{-1} mol^{-1} is the constant of universal gas and T is the absolute temperature (K).

The free energy of activation (ΔG*) was determined at different temperatures from the relationship shown in the following equation

$$\Delta G^* = -RT \ln\left(\frac{Kdh}{kT}\right)$$

where h is the Planck's constant (6.626×10^{-34} J·s), k is the Boltzmann one (1.381×10^{-23} J·K^{-1}) and Kd can be defined as the following equation

$$Kd = \left(\frac{KT}{H}\right) e^{\left(\frac{\Delta H^*}{RT}\right) e^{(\Delta S^*/R)}}$$

Activation entropy (ΔS*) was calculated using the formula shown in the next Equation

$\Delta S^* = (\Delta H^* - \Delta G^*)/T$

The enzyme half-time (t1/2), as specified by Gohel and Singh [19], was defined as the time after which the activity of the enzyme had been lowered to half of its original activity and then estimated according to the equation:

$t_{1/2} = \ln 2/Kd$

D-value (the decimal reduction time) as described by Pal and Khanum, was defined as the time of enzyme exposure at a specified temperature to preserve a remaining activity of 10% [20].

$D = 2.303/Kd$

Kinetic constant (K_m)

Lineweaver-Burk plots were generated using polygalacturonic acid concentrations from 1.5-3.5 mg /0.5 mL to determine the K_m values.

Effect of metal cations

The enzyme was incubated with 5 mM solutions of Ca^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}, Ni^{2+}, Pb^{2+}, Cd^{2+} and Hg^{2+} for 15 min before the addition of the substrate. Enzyme activity without the metal cation is considered to be 100% activity. The relative enzyme activity in the presence of each metal cation was determined.

Orange juice clarification by purified enzyme

The fruit juice clarification studies were performed previously described [21]. One hundred µL of exo-polygalacturonase were added to 2 mL of Orange, Apple, Grape, and Peach juices and the reaction mixture was incubated at 37°C for 2 h. Reaction mixtures were incubated in boiling water for 5 min to stop the reaction. In addition, the reaction mixture was centrifuged for 5 min at 3000 rpm. The percentage of transmittance of the reaction mixture was determined at 600 nm compared to controls that contained the same volume of enzyme added before the reaction mixture was boiled.

Result and Discussion

PG produced by *T. pseudokoningii* using citrus Orange peel during solid state fermentation was purified in two steps, including ion exchange chromatography and gel filtration. The purification scheme is summarized in Table-1. The elution profile of the exo-PG from the ion exchange chromatography (DEAE–Sepharose) clearly shows five peaks (Fig. 1), the negatively adsorbed fraction and the fractions eluted with 0.05, 0.1, 0.2 and 0.3 M sodium chloride. The peaks were designated PGP1, P2, P3, P4 and P5. All the protein peaks were assayed for exo-PG activity using PGA as the substrate as described in the Methods. Exo-PGP4 had the highest activity and was purified further on a Sephacryl S-200 gel filtration column. The final exo-PGP4a obtained (Fig. 2A) had the highest specific activity of 1208 unit/mg protein with 20-fold purification. The molecular mass of the purified exo-PGP4a was determined to be 25 kDa using Sephacryl S-200 column. In addition, the purity of the protein was also determined using SDS-PAGE, which showed a single band of protein at 25 kDa (Fig. 2B). Our finding is consistent with previous studies, which showed that the molecular masses of PG ranged from 24 to 34 kDa and were detected in *Penicillium Expansum* [22], *Penicillium Viridicatum* [23], and *Aspergillus Awamori* [24]. In addition, we determined that the purified exo-PGP4a showed maximum PG activity at pH 6 (Fig. 3A), similar to the result obtained by Esawy [1]. Acidic pH optima ranging from 4.5 to 6.0 were reported for PG

from *Aspergillus Awamori* (pH 4.5) [24], and rice-weevil (pH 5.5) [25]. Subsequently, we also investigated the effect of temperature on the activity of exo-PGP4a. Exo-PGP4a exhibited a temperature optimum of 45°C (Fig. 3B). Earlier, broad temperature optima (37-40°C) were detected in ripe mango polygalacturonase [26]. A temperature optimum at 50 °C was also reported for the polygalacturonase produced by *Penicillium Notatum* [27]. In understanding structure-function interactions, thermodynamic research of the enzyme at greater temperatures is of excellent importance. ΔH* and ΔS* are two significant parameters relating to the enzyme structure function and the numerical values of these two parameters are defined by the structure and solvent effects [28]. Enthalpy offers an estimate of the number of non-covalent bonds broken, while entropy measures the net quantity of enzyme and/or solvent disorders in the transition state. Table-2 displays the results of thermodynamic parameters for exo-PG. At 55 and 75 ° C, the ΔH* values for exo-PGP4a were 39.91 and 39.74 kJ / mole, indicating the labile nature of exo-PGP4a, and decreased ΔS* values with greater temperatures were potentially due to the aggregation and compaction of the partly unfolded enzyme at greater temperatures, which predominates at greater temperatures during protein exposure [29, 30]. ΔH* and ΔS* provide data on the amount of broken non-covalent bond (mainly H-Bond) and thermal inactivation-related net solvent-enzyme disorder. High-activation enthalpy values (ΔH*) are characteristic of protein denaturation [30], and thermo-stabilization of proteins is mostly followed by a decline in ΔS* and ΔH* [31]. The increase in Gibbs free thermal inactivation energy (ΔG*) with rising temperature was due to heat unfolding resistance owing to the intrinsic contribution of enzyme molecules 'polypeptide chains'. At 50 and 75 °C, ΔG* values were observed to be 90.86 and 94.18 kJ/mol. The decimal reduction time (D value) is a parameter commonly used in the evaluation of enzyme stability. D values for exo-PGP4a ranged between 169.33-70.86 min. The decimal reduction D value, at different temperatures (55-75°C) is shown in Table-2. Stability of enzyme improves with the increase of D value. In addition, to elucidate the kinetics of exo-PGP4a enzymatic activity, we determined the kinetic parameters of the exo-PGP4a using the Lineweaver-Burk plot (Fig. 3C). We used purified exo-PGP4a to determine the K_m value for the hydrolysis of PGA and found it to be 2 mg/ml. The V_{max} was 3.27µmol min^{-1} mg^{-1}, while K_m/V_{max} was 0.612. A low value of Km suggests a better affinity for the substrate. Previous studies on the exo-PG produced by *Aspergillus flavus* and *Mucor circinelloides* found K_m values of 2.08 and 2.2 mg/mL, respectively [32, 33]. In addition, we studied the effect of metal cations on exo-PGP4a at 5 mM concentration in the reaction mixture (Table-3). We found that among all the metal cations studied, Cu^{2+} and Zn^{2+} enhanced the activity of exo-PGP4a, while Pb^{2+}, Ca^{2+} and Ni^{2+} partially inhibited the enzyme. Interestingly, the purified enzyme was strongly inhibited by Co^{2+}, Cd^{2+} and Hg^{2+}. In addition, metal cations, such as Cu^{2+} have been reported to enhance the activity of exo-polygalacturonase from *Aspergillus Flavus*, which was moderately inhibited by Ca^{2+}, Co^{2+} and Zn^{2+} [32]. Similar studies on an acidic endo-PG from *A. niger* found it to be enhanced by Cu^{2+} metal cation (Zhou *et al.*, 2015), while Hg^{2+} ions completely inhibited the PG from *Streptomyces lydicus* [34]. Also shown were inhibitory effects of Co^{2+}, Ni^{2+}, Hg^{2+} and Zn^{2+} ions on the *Penicillium Viridicatum* exo-PG while Ca^{2+} had no effect on exo-PG activity [23]. Fig. 4 illustrates the mode of action of purified PGP4a. The analysis of the hydrolytic products after PGA hydrolysis by purified PGP4a using TLC chromatography showed that monogalacturonic acid was the primary product. This reveals that the purified PG is an exo-PGP4a.

Table-1: Purification scheme for Polygalacturonase.

Recovery 100%	Fold purification	S.A Unit/mg protein	T. Protein mg	T. units*	steps
100	1	60.7	17.3	1050	Crude extract
					Chromatography DEAE-sepharose
11	1	62.6	1.9	119	0.0 M NaCl (PGP1)
12	1.7	105	1.2	126	0.05M NaCl (PGP2)
14	1	62.5	2.4	150	0.1M NaCl (PGP3)
22	2.3	138	1.7	235	0.2M NaCl (PGP4)
13	1.3	81.7	1.7	139	0.3M NaCl (PGP5)
		Gel filtration on sephacryl s-200			
12.6	18	1100	0.12	132	P4a

* One unit of enzyme activity was defined as the amount of enzyme which liberated 1 µmol of galacturonic acid per minute under standard assay conditions.

Table-2: Thermodynamic parameters for the irreversible thermal denaturation of exo-PG from *T. pseudokoningii.*

E_a*kJ/mol	R^2	D values	ΔS (J/mol/k)	ΔG (kJ/mol	ΔH (kJ/mol)	$t_{1/2}$ (min^{-1})	Kd (min^{-1})	Temp. (K)
	0.9854	169.33	-157.74	90.86	39.91	50.95	0.0136	323
	0.9868	207.47	-161.59	92.87	39.87	62.43	0.0111	328
42.596	0.9717	200.26	-163.33	94.22	39.83	60.26	0.0115	333
	0.9012	114.00	-160.68	94.1	39.79	34.30	0.0202	338
	0.9527	70.86	-158.72	94.18	39.74	21.32	0.0325	343

Table-3: Effect of metal ions on the activity of purified exo-PGP4a.

Effect of metals (5 mM)	% Relative activity
Control	100
Ca^{2+}	61
Ni^{2+}	53
Pb^{2+}	75
Co^{2+}	24
Hg^{2+}	14
Cu^{2+}	183
Zn^{2+}	156
Cd^{2+}	8

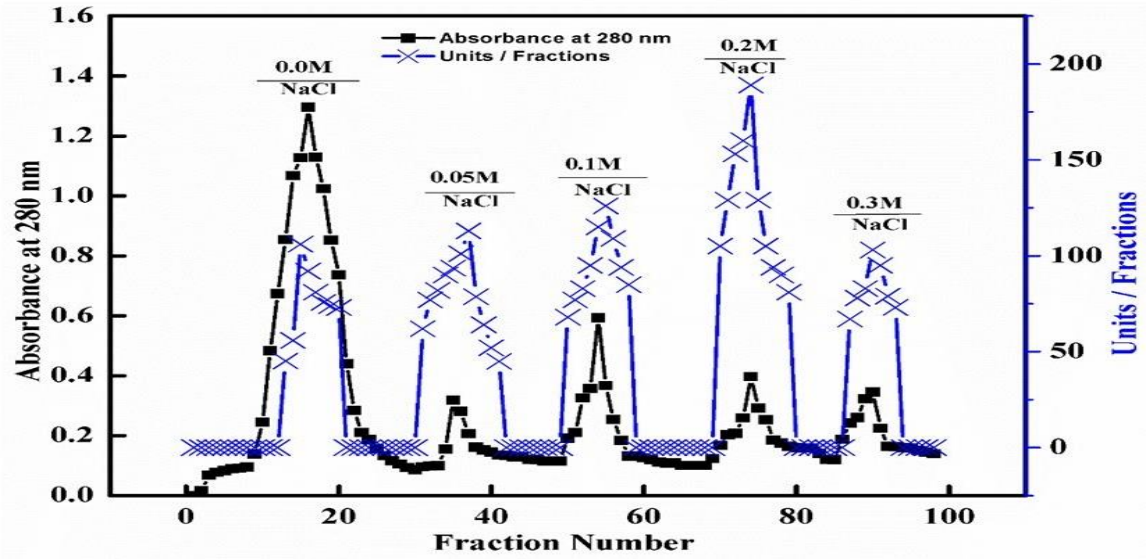

Fig. 1: A typical elution profile for the chromatography of PG using a DEAE-Sepharose column previously equilibrated with 20 mM Tris-HCl buffer, pH 7.2.

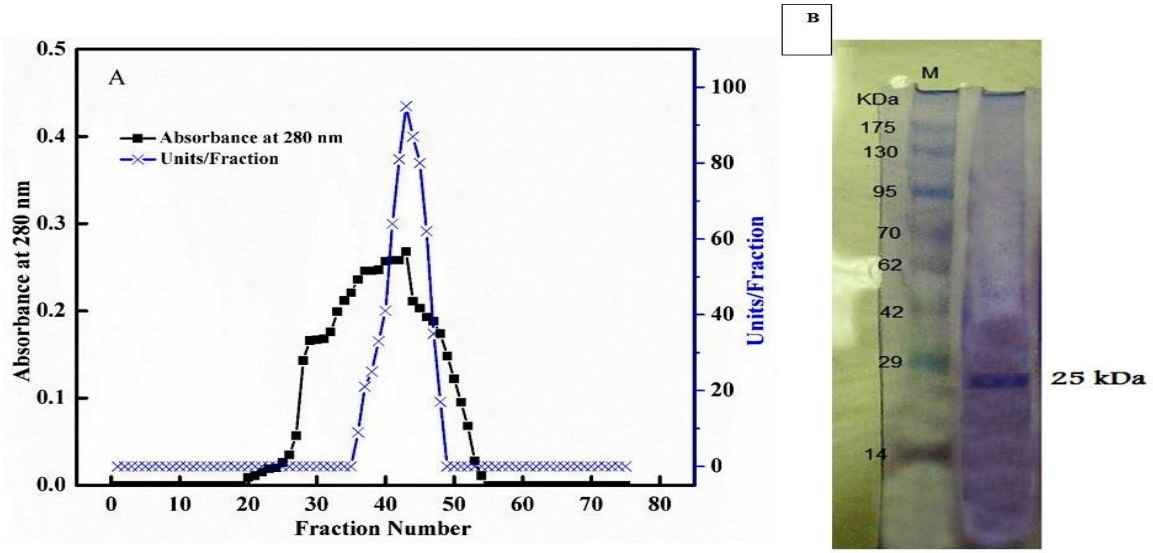

Fig. 2: Gel filtration of PGP4a DEAE-Sepharose fractions using a Sephacryl S-200 column. The column was equilibrated with 20 mM Tris-HCl buffer, pH 7.2 (A), SDS-PAGE for homogeneity and molecular weight determination of PGP4a (B).

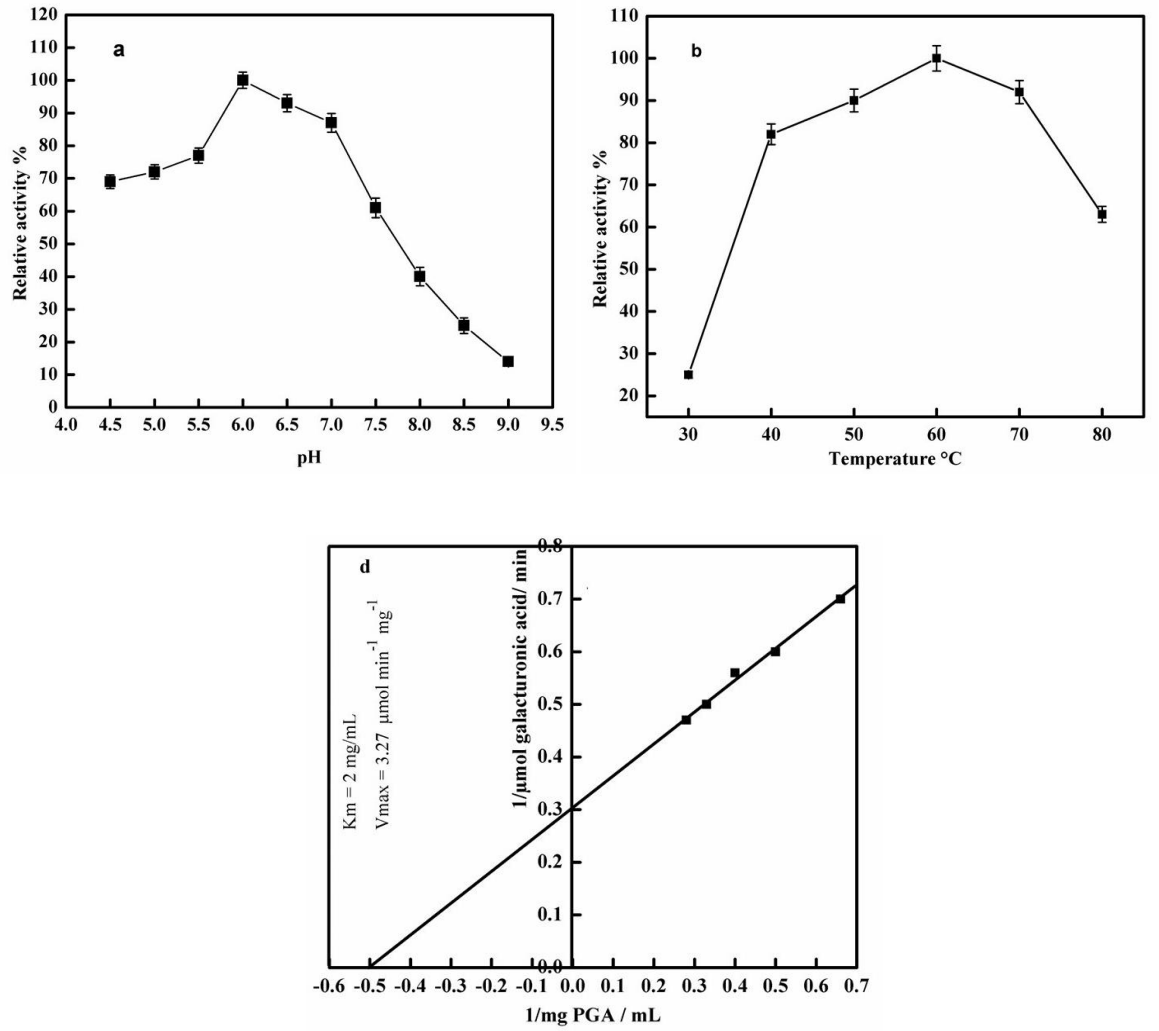

Fig. 3: Optimum pH (a), effect of temperature on activity (b), Kinetics parameters (Km) (c) of purified exo-PG P4a.

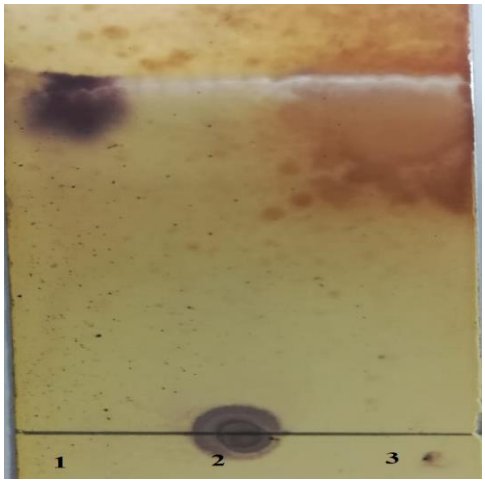

Fig. 4: Thin layer chromatography of purified exo-PGP4a. Lane 1, monogalacturonic acid, Lane 2, polygalacturonic acid, Lane 3, Hydrolytic product of the purified enzyme.

Application of exo-PG in the clarification of fruit juice

Colloid formation is the primary problem faced by the fruit juice industry due to high concentrations of pectin. In this study, we examined the enzymatic properties of exo-PG from *T. Pseudokoningii* and found it to be suitable for the industrial application of fruit juice. In this study, we examined the clarification of fruit juice using the purified exo-PG. We obtained Orange, Apple, Grape, and Peach juices from a local market and studied the effect of the purified PG from *T. Pseudokoningii*. We found that the transmittance increased by 21%, 28%, 17.5% and 31% for Orange, apple, grape and peach, respectively compared to the control (Fig. 5). We hypothesized that the transmittance of the treated juice increased due to the degradation of pectin, which produces the majority of colloidal and suspension particles in the juice. The degradation of pectin with the Pectinases present in fruit juice led to a reduction in viscosity and aggregate formation. This resulted in more concentration of the juice, which was clear in flavour and colour [35,36]. Polygalacturonases have proven to benefit the food industries and are added to clarify vegetable or fruit juices. Various studies on different fungal species, such as *Aspergillus Carbonarius* and *Achaetomium sp.*Xz8 have led to the isolation of several polygalacturonases that have been shown to improve the clarity and juice yield [35], *Aspergillus Flavus* [32], help in reduce the viscosity of papaya juice [37].

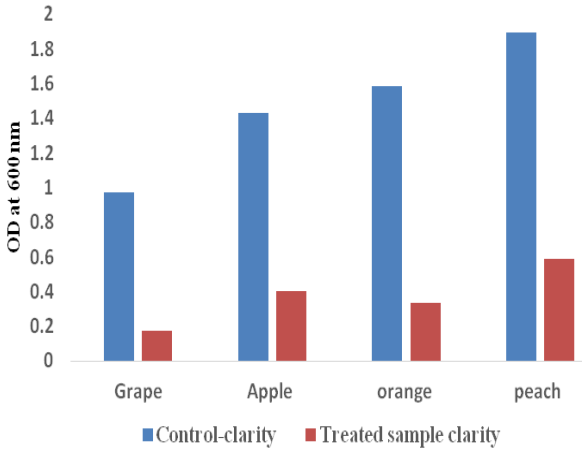

Fig. 5: Effect of exo-PGs on clarity of fruit (grape, apple, Orange and peach) juices.

Conclusion

In Conclusions, we purified and characterized the PG from *T. Pseudokoningii* biochemically. We investigated the role of exo-PG for fruit juice clarification based on its acidic nature. The purified enzyme (exo-PGP4a) has a relative molecular mass of 25 kDa. We found that the exo-PGP4a has an optimum pH of 6.0 and an optimum temperature of 45°C. The thermostability profile of exo- PGP4a was considerably interesting and followed biphasic deactivation kinetics. Several metal cations such as Cu^{2+} activated the enzymatic activity, while Co^{2+}, Pb^{2+}, Ca^{2+}, Ni^{2+}, Cd^{2+} and Hg^{2+} inhibited it. In summary, our research showed that exo-polygalacturonase from *T. Pseudokoningii* is a good potential candidate for application in the fruit juice industry.

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