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Characterization of three-phase partitioned exo-polygalacturonase from *Aspergillus sojae* with unique properties

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Abstract

Exo-polygalacturonase enzyme produced by *Aspergillus sojae ATCC* 20235 was purified using three-phase partitioning (TPP), an emerging bio-separation technique where a single step as compared to the classical multi-step purification was used. Using this technique, crude enzyme solution (pH 6.6) saturated to 30% (w/v) with ammonium sulphate and with a crude extract to *tert*-butanol ratio of 1:1 (v/v) at 25 °C resulted in 25.5% recovery of exo-polygalacturonase with a 6.7-fold purification. The purified enzyme was characterized with respect to its activity and stability at various pH and temperature ranges. Optimum pH and temperature for maximum activity were determined as pH 4 and 55 °C. The enzyme was stable at both acidic and alkaline pH for 2 h at 30 °C. The thermal stability study showed that the purified enzyme had an inactivation energy of 68.41 kcal/mol and a half-life ($t_{1/2}$) value of 3.6 h at 75 °C presenting a large thermal stability. The kinetic constants K_m and V_{max} using polygalacturonic acid as substrate were 0.75 g l^{-1} and 1.14 μmol min^{-1}, respectively. SDS-PAGE profiling revealed that the purified exo-polygalacturonase had two bands with the molecular weights of 36 and 53 kDa. The enzyme was completely inhibited in the presence of Mn^{2+} and SDS and induced significantly by EDTA, glycerol and β-mercaptoethanol.
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Keywords: Exo-polygalacturonase; Three-phase partitioning; *Aspergillus sojae*; Microbial enzymes; Enzyme characterization

1. Introduction

Microbial pectolytic enzymes of fungal origin are of great importance with tremendous potentials to offer to the industry. Among these applications are retting of flax and vegetable fibres, increasing the yield of fruit juice extraction, de-pectinisation and clarification of fruit juices, extraction of oils from vegetables and citrus peels, pre-treatment of pectic waste water, fermentation of coffee and tea, manufacturing of paper and pulp, production of poultry feed and purification of plant viruses [1,2]. It is reported that pectinases hold a share of 25% in the global sales of food enzymes where this value is expected to increase over time with the invention of new application areas [3]. Pectolytic enzymes are classified mainly into two main groups of de-esterification and depolymerising enzymes based on their degradation mechanism [1,4,5]. The enzymes depolymerising polygalacturonic acid are further classified into polygalacturonase simply hydrolyzing the α-1,4-linkage and polygalacturonate lyase cleaving *trans*-eliminatively. Both include exo-type enzymes, which catalyze the release of single galacturonic acid residue from the non-reducing end and random fashion endo-type hydrolyzing enzymes which release oligosaccharidic chains of various lengths [1].

Since applications of pectinases in various fields are broadening, it is important to discover new strains producing pectinases with novel properties and understand the nature and properties of these enzymes for their efficient and effective usage. With this perspective exo-polygalacturonase which attracts the most attention due to its wide use among pectinases is produced in this study by *Aspergillus sojae ATCC* 20235 (not considered for this purpose so far) and purified by three-phase partitioning (TPP) for a one-step purification based on a modified procedure reported by Sharma and Gupta [6]. In this process (TTP) the protein extract is mixed with ammonium sulphate and *tert*-butanol. Three phases are formed within short periods of time (about 1 h): lower aqueous layer, interfacial protein precipitate and upper *tert*-butanol layer [7]. This technique is an emerging bio-separation technique, which employs collective operation of

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principles involved in numerous techniques such as conventional salting out, cosolvent, isoionic, osmolytic and kosmotropic precipitation of proteins [8].

The purified enzyme was further characterized with respect to its activity and stability at various pH and temperature ranges. SDS-PAGE profiling and determination of its K_m and V_{max} parameters together with its thermal inactivation kinetics was carried out. The effect of metal ions and certain compounds such as EDTA, glycerol, Tween 80, SDS and β-mercaptoethanol were also investigated. To date there is no literature report on the purification and characterization of this enzyme using this organism. This paper will be one of the initial studies in this field. In our previous work [9–11] we reported that this organism holds a great potential for the production of this enzyme in submerged and solid state fermentation. Therefore in order to exploit new industrial potentials of exo-polygalacturonase it is necessary to investigate new microbial strains and understand the structure–stability relationship of this enzyme. Separation and purification of proteins/enzymes, which account for a major fraction of the overall production cost, in an efficient way and maintaining the desired level of enzyme activity over a long period of time and improving its stability are important parameters for the selection and design of pectinases [5,8]. The knowledge gained in this way will offer new possibilities in improving the potential and effective use of this enzyme in such diverse and broad areas.

2. Materials and methods

2.1. Microorganism and development of inoculum

A. sojae ATCC 20235 was purchased in the lyophilized form, from Promochem Inc., an international distributor of ATCC (American Type of Culture Collection) in Europe. YME agar slant medium containing, malt extract (10 g/l), yeast extract (4 g/l), glucose (4 g/l) and agar (20 g/l) was used for the propagation of this culture and incubated at 30 °C until well sporulation. Stock cultures of these strains were prepared with 20% glycerol water and stored at −80 °C.

The spore suspensions used as inoculum were obtained on molasses agar slants containing glycerol (45 g/l), peptone (18 g/l), molasses (45 g/l), NaCl (5 g/l), FeSO$_4$·7H$_2$O (15 mg/l), KH$_2$PO$_4$ (60 mg/l), MgSO$_4$ (50 mg/l), CuSO$_4$·5H$_2$O (12 mg/l), MnSO$_4$·H$_2$O (15 mg/l) and agar (20 g/l), after the pre-activation step performed on YME agar using the stock cultures. The incubation temperature and time for each of the steps were 30 °C and 1 week, respectively. The harvesting of the spores from the slants was done using 5 ml of Tween80-water (0.02%). The spore suspension was collected in sterile falcon tube and stored at 4 °C until the actual study. The initial spore counts and viability counts were recorded.

2.2. Production of exo-polygalacturonase

The cultivation was carried in a solution containing glucose (25 g/l), peptone (2.5 g/l), disodium phosphate (3.2 g/l), monosodium phosphate (3.3 g/l), maltrin (120 g/l) and corn steep liquor (4 g/l). The fermentation was conducted at 350 rpm at 30 °C for 96 h. After 96 h of incubation, each flask was assayed for enzyme activity and protein determination. Crude enzyme activity was determined on supernatant obtained after the centrifugation of the broth at 5000 rpm for 15 min.

2.3. Three-phase partitioning of the exo-polygalacturonase

Purification of the exo-polygalacturonase enzyme was performed by three-phase partitioning for a one-step purification step based on a modified procedure reported by Sharma and Gupta [6]. In order to adopt the mentioned procedure to our study, the crude enzyme solution (20.4 U/ml at pH 6.6) was brought to 25, 30 and 40% (w/v) saturations using ammonium sulphate (Table 1). To this solution, *tert*-butanol was added in order to obtain 1:1 or 1:2 (v/v) ratio of crude enzyme to *tert*-butanol at the specified temperature (Table 1). After incubation for 1 h at 25 or 37 °C, the mixture was centrifuged (2000 × *g* for 5 min) to facilitate separation of phases. The lower aqueous layer and the interfacial precipitate were collected; the latter was dissolved in 0.1 M sodium phosphate buffer, pH 6.6. The best conditions which resulted into maximum recovery were used as standard purification procedure. The purified enzyme obtained under these conditions was used for further characterization. The activity of the crude extract (20.4 U/ml) initially added was taken as 100%. The time of treatment (incubation time) was not considered as factor variable since in a preliminary study it was determined that 1–2 h did not cause a significant increase, hence 1 h was applied.

2.4. Enzyme assay

Exo-polygalacturonase activity was assayed according to the procedure given by Panda et al. [12] by using polygalacturonic acid as substrate at pH 6.6 and 26 °C. In this study, one unit of enzyme activity was defined as the amount of enzyme that catalyses the release of 1 μmol of galacturonic acid per unit volume of culture filtrate per unit time at standard assay conditions. Galacturonic acid (Sigma, St. Louis, MO) was used as standard for the calibration curve of exo-polygalacturonase activity.

2.5. Protein determination

The total protein content of the samples were determined according to the method described by Lowry et al. [13]; the protein standard used was bovine serum albumine.

2.6. SDS-PAGE profiling

SDS-PAGE was done according to the procedure reported by Laemmli [14] using 10% resolving and 5% stacking gel. Protein bands were visualized after staining with Coomassie brilliant blue R250 for 1 h and de staining using methanol–water containing 10% acetic acid for 2–3 h. The molecular weight of the protein bands were identified using Gel Documentation System

Table 1
Various conditions used for three-phase partitioning (TPP) of the exo-polygalacturonase enzyme

Condition	Temperature (°C)	Percent saturation with ammonium sulphate (w/v)	Enzyme:*tert*-butanol ratio (v/v)	Degree of purification	Activity recovery (%)
1	25	25	1:1	1.1	12.7 ± 2.4
2	25	25	1:2	0.5	7.4 ± 5.9
3	25	30	1:1	6.7	25.5 ± 1.8
4	25	30	1:2	1.5	7.8 ± 3.2
5	25	40	1:1	1.7	10.3 ± 3.3
6	25	40	1:2	3.4	17.2 ± 1.3
7	37	25	1:1	0.1	1.0 ± 0.3
8	37	25	1:2	0.4	3.4 ± 0.8
9	37	30	1:1	0.7	8.8 ± 1.4
10	37	30	1:2	1.2	9.8 ± 1.4
11	37	40	1:1	2.8	21.6 ± 4.7
12	37	40	1:2	1.5	9.8 ± 1.5

TPP was carried out after the pH of the exo-polygalacturonase enzyme preparation had been adjusted to 6.6. The starting volume of the enzyme was 2 ml under each condition.

(CN-3000 WL) and the associated software Bio-1D++, Vilber Lourmat (France).

2.7. Effect of pH on activity and stability of the purified exo-polygalacturonase

The effect of pH on the activity of the purified exo-polygalacturonase was determined by assaying the enzyme activity at different pH values ranging from 3.0 to 12.0 using 0.1 M of the following buffer systems: acetate (3.0, 4.0, and 5.0), phosphate (pH 6.0 and 7.0), tris–HCl (pH 8.0, 9.0, and 10.0) and Na_2HPO_4–NaOH (pH 11.0 and 12.0) buffer systems [15]. The relative activities were based on the ratio of the activity obtained at certain pH to the maximum activity obtained at that range and expressed as percentage.

The pH stability of *A. sojae* exo-polygalacturonase was investigated in the pH range of 3.0–12.0. Therefore, 1 ml of the crude enzyme was mixed with 1 ml of the buffer solutions mentioned above and incubated at 30 °C for 2 h. Afterwards aliquots of the mixtures were taken to measure the residual exo-polygalacturonase activity (%) with respect to control, under standard assay conditions.

2.8. Effect of temperature on activity and stability

The effect of temperature on the activity of the purified exo-polygalacturonase was determined by performing the standard assay procedure at different temperatures ranging from 25 to 75 °C (25, 37, 45, 55, 65, and 75 °C). Before the addition of enzymes, the substrate (0.24% (w/v) polygalacturonic acid) was preincubated at the respective temperature for 10 min. The relative activities (as percentages) were expressed as the ratio of the purified exo-polygalacturonase activity obtained at certain temperature to the maximum activity obtained at the given temperature range.

The thermostability of the purified exo-polygalacturonase was investigated by measuring the residual activity after incubating the enzyme at various temperatures ranging from 25 to 65 °C (25, 37, 45, 55 and 65 °C) for 10, 20, 30 and 60 min.

2.9. Kinetics of thermal inactivation and estimation of the inactivation energy

Thermal inactivation kinetics of the purified exo-polygalacturonase was studied by incubating the enzyme at different temperatures (75, 80, 82.5 and 85 °C) in the absence of the substrate. Aliquots were withdrawn at periodic intervals and cooled in an ice bath prior to assay as described above. The residual activity was expressed as % of the initial activity. From a semilogarithmic plot of residual activity versus time, the inactivation rate constants (k_d) were calculated (from slopes), and apparent half-lives were estimated using Eq. (1). The half-life is known as the time where the residual activity reaches 50%.

$$t_{1/2} = \frac{\ln 2}{k_d} \tag{1}$$

The temperature dependence of k_d was analyzed using the Arrhenius plot [16]. The inactivation energy was calculated from the Arrhenius equation as

$$\ln(k_d) = \ln(k_0) - \left(\frac{E_d}{R}\right)\frac{1}{T} \tag{2}$$

The values of E_d and k_0 were estimated from the slope and intercept of the plot of $\ln(k_d)$ versus $1/T$, respectively. (R is the ideal gas constant = 1.987 cal mol^{-1} K^{-1}).

2.10. Determination of kinetic constant

The kinetic constants (V_{max} and K_m) were determined using Lineweaver–Burk double reciprocal ($1/v$ versus $1/S$) plot [16], where different concentrations of polygalacturonic acid were used as substrate (0.1–1 mg/ml) at pH 6.6 and 26 °C.

$$\frac{1}{v} = \frac{1}{V_m} + \frac{K_m}{V_m}\frac{1}{S} \tag{3}$$

With a slope of K_m/V_m and intercept of $1/V_m$ (Eq. (3)) estimated K_m and V_m values were determined.

All the analytical grade materials and the culture media ingredients were obtained from Sigma–Aldrich.

2.11. Effect of metal ions and various compounds on the exo-polygalacturonase activity

Metal ions (K^+, Na^+, Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+} and Fe^{2+}) and various compounds (EDTA, glycerol, Tween 80, SDS and β-mercaptoethanol) were tested for their effect on purified exo-polygalacturonase activity in Na-phosphate buffer, pH 6.6. The enzyme was preincubated for 30 min at 37 °C with 1 mM, 5 mM and 10 mM of listed ions and compounds (Tables 3 and 4) as a final concentration prior to the substrate addition. Activity without added metal ions and compounds was taken as 100% activity. Each value represents the average of two experiments. The confidence limit used in all experiments was 95%.

3. Results and discussion

3.1. Three-phase partitioning of the exo-polygalacturonase

In order to adapt the procedure developed by Sharma and Gupta [6], to our study, various experiments at different percent saturations of ammonium sulphate, different crude extract to *tert*-butanol (v/v) ratio and different temperatures as given in Table 1 were performed. In all the experiments the starting protein concentration (containing 20.4 U ml^{-1} of exo-polygalacturonase activity) was 15.6 mg ml^{-1}. The effect of these parameters on the yield and on the degree of purification is presented in Table 1. As it is seen all these three parameters have major influence on the purification of the exo-polygalacturonase. For example an increase of the saturation level of ammonium sulphate from 25 to 30%, at 1:1 crude extract to *tert*-butanol (v/v) ratio and 25 °C, increased the recovery by 2 and purification by 6.1-fold, however, further increase to 40% saturation decreased the same responses by 2.5 and 3.9, respectively. Similarly, an increase of the temperature from 25 to 37 °C at 30% saturation of ammonium sulphate and 1:1 crude extract to *tert*-butanol (v/v) ratio caused 2.89- and 9.57-fold of reduction in recovery yield and purification, respectively. The effect of the crude extract to *tert*-butanol (v/v) ratio was also very considerable where the change from 1:1 to 1:2 crude extract to *tert*-butanol (v/v) ratio, decreased the responses (recovery and degree of purification) by 3.26 and 4.47, respectively. Based on these observations the best results were obtained at 30% saturation with ammonium sulphate, 1:1 (v/v) ratio of crude extract to *tert*-butanol and 25 °C where, 6.7-fold purification and 25.5% recovery was observed. The purified enzyme in the forthcoming characterization experiments was obtained by this adapted procedure. These conditions were in good correlation with various applications of TPP on different proteins, enzymes and soybean oil [17–20]. It is reported that working with high concentrated protein solutions often leads to loss of yields, where poor results are obtained beyond 10 mg ml^{-1} of the starting protein concentration [20,21]. Therefore this might be one of the reasons in this study for the low recovery yield (25.5%). It is recommended in future studies to start with lower protein concentrations. However, the
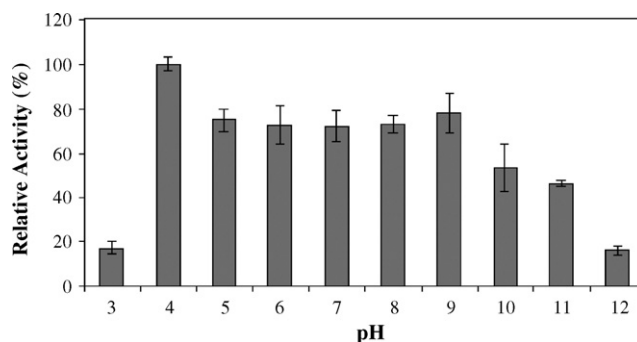

Fig. 1. Effect of pH on the activity of the purified exo-polygalacturonase enzyme. Each experiment was carried out three times, the difference in individual readings in each set of corresponding experiments was less than 10.5%.

degree of purification of exo-polygalacturonase obtained in this study was 1.68, 3.52 and 5.73 times higher than the studies conducted by Keon and Waksman, Riou et al. and Sakamoto et al., respectively, where ammonium sulphate precipitation was used alone [22–24]. In those studies ammonium sulphate precipitation serves as an intermediate step prior to other chromatographic steps used in the purification, however, here a pure enzyme not requiring any other treatment is obtained in a single step. Therefore this treatment is economically more feasible and relatively simple. Besides when the degree of purification was compared to various studies where TPP was employed, the current results were relatively above those [7,8,21,25]. It was concluded that this modified procedure could be recommended as a single purification step of exo-polygalacturonases of similar organisms and holds a potential to be applicable to industrial scales.

3.2. Effect of pH on exo-polygalacturonase activity and stability

The effect of pH on the exo-polygalacturonase activity is presented in Fig. 1. As it can be observed, the enzyme was active over a broad pH range, displaying over 71% of its activity in the pH range of 4.0–9.0 with an optimum pH of 4. A slight increase to 78% relative activity was observed at pH 9. A further distance from these pH values decreased the relative activity down to 17%. These findings were in correlation with several earlier reports showing pH optima of 4.8 for exo-polygalacturonase from *A. niger* and pH optima of 5.0 for *Rhizopus stolonifer* [5]. Similarly, the polygalactururonase of *S. thermophile* Apinis was reported to be active over a wide pH range (3.0–9.0) as well [26]. Among the polygalacturonases obtained from different microbial sources, most have the optimal pH ranges of 3.5–5.5 [3]. The high relative activity in the alkali pH region in addition to the acidic pH optima, will open up new application windows for the current purified exo-polygalacturonase unlike other exo-polygalacturonases which are mostly known to have narrow pH ranges [3].

Investigations on pH stability by incubating the enzyme in suitable buffer systems for 2 h at 30 °C (Fig. 2) revealed, that the exo-polygalacturonase enzyme was very stable at the broad pH range of pH 4–9, retaining more than 73% of its activity. At
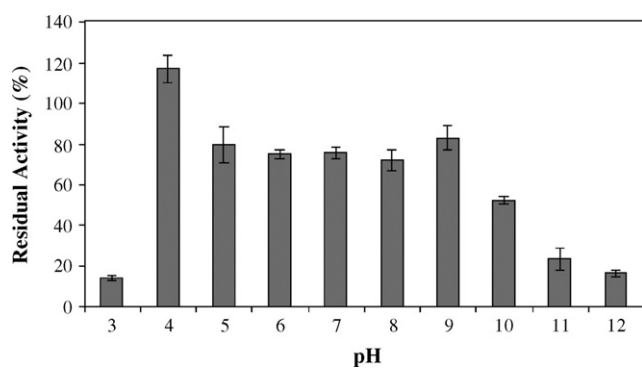
Fig. 2. Effect of pH on the stability of the purified exo-polygalacturonase enzyme. Each experiment was carried out three times, the difference in individual readings in each set of corresponding experiments was less than 8.7%.

Fig. 4. Effect of temperature on the stability of the purified exo-polygalacturonase enzyme. Each experiment was carried out two times, the difference in individual readings in each set of corresponding experiments was less than 15%.

its optimum pH of 4 the residual activity was 17.5% higher than the control. However, the stability was significantly reduced to 52–16% between pH 10 and 12. It is reported that the inactivation process is found to be faster at high alkaline pHs due to disulfide exchange, which usually occurs at near neutral and alkaline conditions.

Therefore, exo-polygalacturonase like the one characterized in this study can be a potential candidate for different applications in the industry demanding either acidic or alkaline pH ranges. In addition, with the broad pH stability range, this enzyme can be considered suitable for applications requiring long exposure times. Hence, enhancing the stability and maintaining the desired level of activity over long period of time are two significant points which are taken into account for the selection of enzymes in industrial applications.

3.3. Effect of temperature on exo-polygalacturonase activity and stability

The *A. sojae* exo-polygalacturonase was active over a broad temperature range of 25–75 °C with an optimum temperature of 55 °C (Fig. 3). It retained more than 66% of its activity at 65 and 75 °C. This optimum temperature was in agreement with the studies conducted by several authors using different strains. For example Sakamoto et al. [22], found the optimum temperature
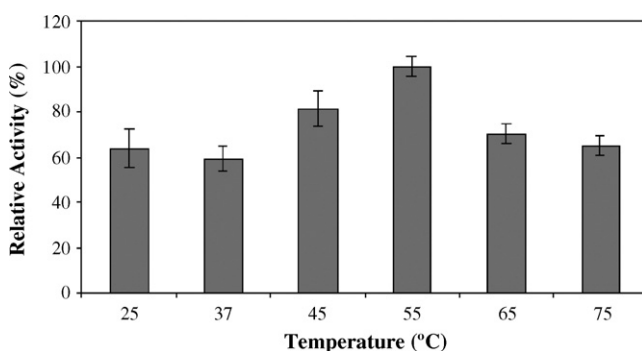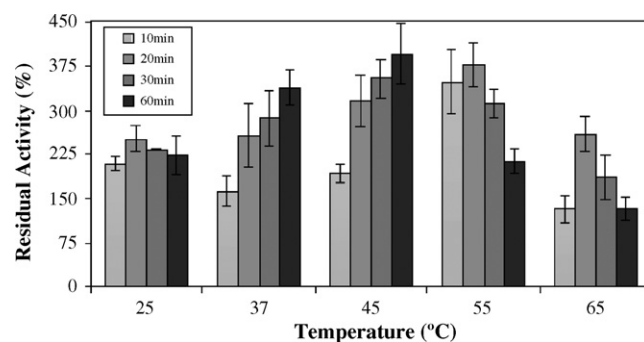

Fig. 3. Effect of temperature on the activity of the purified exo-polygalacturonase enzyme. Each experiment was carried out three times, the difference in individual readings in each set of corresponding experiments was less than 8.2%.

of an exo-polygalacturonase from *A. niger* as 60 °C, similarly, maximum activity for commercial enzyme, Rapidase C80 was determined at 55 °C [27]. Furthermore, the partially purified polygalacturonase from *Sporotrichum thermophile Apinis* was optimally active at 55 °C as well [3,4].

Thermostability is the ability of enzyme to resist against thermal unfolding in the absence of substrates [28]. The thermostability of the purified exo-polygalacturonase was determined by measuring the residual activity of the enzyme after incubation at various temperatures ranging from 25 to 65 °C for 10, 20, 30 and 60 min. As it is shown in Fig. 4, the increase in temperature and time caused an overall increase in the stability until 55 °C and 20 min after which a decline was observed. The maximum residual activity of 397% was observed at 45 °C and 60 min. The enzyme was very thermostable even at 65 °C incubated for 60 min, it possessed 133% of residual activity. This clearly demonstrated that the enzyme was induced with the temperature increase and especially at its optimum temperature of 55 °C incubated for 20 min it was stable and exhibit 379% of residual activity. This is an important finding pointing out that in various applications carried out at this temperature the total enzyme amount required will be reduced significantly which will be reflected eventually in the economics of the process. In order to increase productivity and reduce microbial contaminations, industrial processes may require reactions carried out at high temperatures. That is why thermostable enzymes have been the target of many studies involving the elucidation of thermal inactivation mechanism and development of strategies for stability enhancement [27]. Therefore, the current exo-polygalacturonase in this study will exhibit a new example for thermostable enzymes obtained from a new source (*A. sojae*) with unique properties.

After the use of exo-polygalacturonases in the industry it is important to remove or inactivate them, where the estimation of thermal inactivation comes in to the picture discussed below.

3.4. Kinetics of thermal inactivation and estimation of the inactivation energy

Enzyme inactivation is one of major constrains in biotechnological processes therefore it is important to understand the

Table 2

Kinetic parameters for thermal inactivation of the purified exo-polygalacturonase enzyme

T (°C)	k_d (min^{-1})	$t_{1/2}$ (min)
75	0.0032	216.609
80	0.0285	24.321
82.5	0.0350	19.801
85	0.0492	14.088

From slope of the semilogaritmic plot of residual activity vs. time for each temperature, the inactivation rate constants (k_d) were calculated. ($R^2 = 0.98$ for 85 °C, $R^2 = 0.93$ for 82.5 °C, $R^2 = 0.95$ for 80 °C and $R^2 = 0.72$ for 75 °C).

Table 3

Effect of metal ions on the activity of the purified exo-polygalacturonase enzyme

	% Relative activity	
Control	100	
Metal ions (mM)	1	10
KCl	174.81 ± 3.95	94.35 ± 0.91
NaCl	124.57 ± 3.67	84.75 ± 3.19
CaCl$_2$	116.00 ± 2.58	101.25 ± 3.26
MgCl$_2$	93.51 ± 4.01	93.42 ± 3.93
MnCl$_2$	0 ± 0	0 ± 0
ZnCl$_2$	99.94 ± 1.12	112.79 ± 1.83
CuSO$_4$	55.47 ± 2.07	42.67 ± 3.17
FeSO$_4$	74.58 ± 2.20	97.66 ± 1.10

Activity without added metal ions was taken as 100% activity.

mechanism of inactivation. Inactivation is defined as a process where the secondary, tertiary or quaternary structure of a protein changes without breaking covalent bonds. The inactivation of pectolytic enzymes is assumed to follow first order kinetics [29]. Inactivation rate constants (k_d) of exo-polygalacturonase which are presented in Table 2 (at 75, 80, 82.5 and 85 °C) were calculated from the slope of semilogarithmic plot of residual activity versus time. Similarly the half-life values estimated using these constants and Eq. (1), are presented in the same Table. The half-life of exo-polygalacturonase at 75 °C was 8.9, 10.96 and 15.36 times higher than the half-life values at temperatures of 80, 82.5 and 85 °C, respectively at pH 6.6. This issue once again reveals the thermal stability of this enzyme at 75 °C and its easy inactivation at higher temperatures such as 80 and 85 °C.

Inactivation energy of purified exo-polygalacturonase was determined as $E_d = 68.41$ kcal mol^{-1} from the slopes of the linear curve plotted by $1/T$ versus $\ln k_d$ using Eq. (2). This value is in the range of the values (40–70 kcal mol^{-1}) estimated for many microbial enzymes [16]. The inactivation energy estimated for this enzyme was 1.96, 1.71 and 1.77 times higher than the commercial exo-polygalacturonases like Rapidase C80, Pectinase CCM and Pectinex 3XL, respectively, compared to a study conducted by Ortega et al. [27]. These close approximations with the current commercial enzymes indicated that the purified enzyme could be a good candidate for various industrial applications.

3.5. Calculation of kinetic constants

The kinetic parameters of *A. sojae* exo-polygalacturonase for hydrolysis toward polygalacturonic acid (PGA) at pH 6.6 and 26 °C were obtained by a typical double reciprocal Lineweaver Burk plot (Eq. (3)). The K_m and V_m values for hydrolyzing PGA were 0.751 g/l and 1.139 μmol/min, respectively. The estimated K_m value which indicates the affinity of the enzyme towards the substrate is 1.79 and 2.13 times lower than the *A. niger* and *Penicillium frequentans* polygalacturonases, respectively using the same substrate [23]. This indicates that the current exo-polygalacturonase has a higher affinity for PGA compared to the other two. Similarly, the current exo-polygalacturonase had 1.38, 1.47 and 1.65 times more affinity towards PGA substrate than the commercial enzymes Rapidase C80, Pectinase CCM and Pectinex, respectively jugged from the K_m values [27]. V_m which is an indication of the catalytic activity of an enzyme is usually desired to be as high as possible, therefore the cur-

rent value obtained in this study was very comparable to many studies in the literature [27,30].

3.6. Effect of metal ions and various compounds on exo-polygalacturonase activity

The effects of metal ions were examined by adding the chlorides of K$^+$, Na$^+$, Ca^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+} and the sulphates of Cu^{2+} and Fe^{2+} at different concentrations to the buffer solution. As it can be observed from Table 3, the enzyme was induced in the presence of 1 mM of K$^+$, Na$^+$, Ca^{2+} ions, and completely inhibited in the presence of Mn^{2+} at both concentrations studied. Furthermore at 1 mM concentration, Cu^{2+} and Fe^{2+} reduced the enzyme activity where Zn^{2+} did not have any profound effect. The effect of these ions were concentration dependent. For example increasing the concentration of K$^+$, Na$^+$, and Ca^{2+} from 1 to 10 mM, reduced the enzyme activity by 1.85-, 1.47- and 1.15-fold, respectively. Similarly, increasing the concentration of Mg^{2+}, Zn^{2+}, Cu^{2+} and Fe^{2+} to 10 mM, resulted in no change in exo-polygalacturonase activity for Mg^{2+}, a 1.3-fold reduction for Cu^{2+} and 1.12- and 1.31-fold of increase for Zn^{2+} and Fe^{2+}, respectively. In various studies reported in the literature different results were obtained depending on the ion used and the source of the exo-polygalacturonase [31,24]. For example in a study conducted by Kaur et al. [26], it was reported that the activity of pectinase was stimulated by Fe^{2+} and Mn^{2+} at both 1 mM and 5 mM and inhibited strongly at 5 mM by Mg^{2+}. These results were partly in agreement with our results. Similarly, the current results were comparable to a study conducted by Mohamed et al. [30]. Overall these results revealed that the exo-polygalacturonase activity does not necessarily depend on the metal ion except for Mn^{2+} and Cu^{2+}, which inhibit and significantly reduce the activity. The effect of metal ions which are concentration dependent could be important, where media formulations are prepared with raw materials of high salt content.

Various compounds such as listed in Table 4 in different concentrations were examined in order to determine their effect on the exo-polygalacturonase activity. ETDA as chealting agent had promoting effect proportional with concentration in the range of 1–10 mM. This finding was reconfirming that exo-polygalacturonase does not strongly depend on metal ions as discussed above, which otherwise would have been inhib-

Table 4
Effect of different compounds on the activity of the purified exo-polygalacturonase enzyme

Compounds	Concentration (mM)	% Relative activity
EDTA	1	132.98 ± 1.25
	5	165.03 ± 1.43
	10	195.26 ± 0.53
Glycerol	1	111.66 ± 1.06
	5	233.58 ± 1.89
	10	283.76 ± 1.68
Tween 80	(0.02%)	96.41 ± 1.97
SDS	(0.1%)	0 ± 0
β-Mercaptoethanol	(0.1%)	363.16 ± 2.03

The activity without added compound was taken as 100% activity. EDTA: ethylenediaminetetraacetic acid; SDS: sodium dodecyl sulphate.

ited in the presence of EDTA. Similarly, glycerol which is mostly used as protective agent in preservation promoted exo-polygalacturonase activity significantly with an increase in the concentration from 1 to 10 mM. Therefore, glycerol can be recommended for preservation of exo-polygalacturonase enzyme during storage. Tween 80 known as surface-active agent and very popular in the fermentation media formulations, slightly inhibited the enzyme activity at a concentration of 0.02%. SDS a protein denaturant, inhibited enzyme activity of the exo-polygalacturonase completely, whereas β-mercarptoethanol increased the enzyme activity by 3.63-fold compared to control suggesting that disulphide linkages do not have a critical role in maintaining an appropriate conformation of the enzyme for its catalytic activity. This finding was partly in agreement with the study conducted by Kaur et al. [26] who determined inhibitory effect of both SDS and β-mercarptoethanol on thermostable exo-polygalacturonase from *S. thermophile* Apinis.

SDS-PAGE analysis of the purified enzyme revealed two single bands with the molecular weights of 36 and 53 kDa, indicating either the presence of two isozymes of exo-polygalacturonase, or the sub-units of a single polygalacturonase or other impurities. This needs to be exploited further in future. Two different exo-polygalacturonases with different molecular weights such as 38 and 61 kDa; 38 and 65 kDa; 63 and 79 kDa were reported for *A. niger*, *A. japonicus* and *P. frequentans*, respectively [3]. Similar, result of two isoforms with the molecular weights of 46 and 47 kDa of pectinesterase was obtained by a study conducted by Hasanuma et al. [32]. Therefore, the current enzyme will be a new example in the literature with this respect.

4. Conclusion

After characterization of the exo-polygalacturonase purified with an emerging bio-separation technique TTP, it was concluded that this enzyme could be a candidate with potential applications primary in food, waste treatment centers, paper and textile industries. As a exo-polygalacturonase from a new strain (*A. sojae*), which was not considered for this purpose so far, will be a new reference point for the enzyme industry and microbiology area and will fill the gap in the literature. As it

is known discovering new enzymes with novel properties is a tedious and difficult task to perform, therefore enzymes from new sources with unique properties such as the one here may have an enormous economic significance which should not be overlooked.

Acknowledgement

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