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Biochemical and thermal characterization of crude exo-polygalacturonase produced by *Aspergillus sojae*

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ABSTRACT

Crude exo-polygalacturonase enzyme (produced by *Aspergillus sojae*), significant for industrial processes, was characterized with respect to its biochemical and thermal properties. The optimum pH and temperature for maximum crude exo-polygalacturonase activity were pH 5 and 55 °C, respectively. It retained 60–70% of its activity over a broad pH range and 80% of its initial activity at 65 °C for 1 h. The thermal stability study indicated an inactivation energy of E_d = 152 kJ mol^{-1}. The half lives at 75 and 85 °C were estimated as 3.6 and 1.02 h, respectively. Thermodynamic parameters, ΔH^*, ΔS^* and ΔG^*, were determined as a function of temperature. The kinetic constants K_m and V_{max}, using polygalacturonic acid as substrate, were determined as 0.424 g l^{-1} and 80 μmol min^{-1}, respectively. SDS-PAGE profiling revealed three major bands with molecular weights of 36, 53 and 68 kDa. This enzyme can be considered as a potential candidate in various applications of waste treatment, in food, paper and textile industries.

1. Introduction

Pectic substances, mainly degraded by pectolytic enzymes, contribute to the firmness and structure of plant cells (Naidu & Panda, 2003). These enzymes are of multiple nature and various forms due to the complex nature of their substrates. Endo-polygalacturonase (PGL, EC 3.2.1.15), exo-PGL (EC 3.2.1.67), pectate lyase (EC 4.2.2.2), pectin lyase (EC 4.2.2.10) and pectin methyl esterase (EC 3.1.1.11) form a consortium of enzymes which are necessary for the hydrolysis of pectin (Gadre, Driessche, Beeumen, & Bhat, 2003). Pectolytic enzymes of fungal origin attract the most attention since they offer tremendous potential to the industry. Some of their applications are in retting of flax and vegetable fibres, de-pectinisation and clarification of fruit juices, extraction of oils from vegetables and citrus peels, manufacturing of paper and pulp and pre-treatment of pectic waste water (Hoondal, Tiwari, Tiwari, Dahiya, & Beg, 2002; Jayani, Saxena, & Gupta, 2005; Moyo, Gashe, Collison, & Mpuchane, 2003; Saito, Takakuwa, & Oda, 2004). Almost all of the commercial preparations of pectinases are produced from fungal sources, mainly from *Aspergillus niger*. In fact microbial pectinases account for almost 25% of the global food enzyme sales (Jayani et al., 2005). It is also observed that applications of pectinases in various fields are increasing, demanding the discovery of new strains producing pectinases with novel properties. Therefore, it is highly important to determine these characteristics for an efficient application.

Hence, the objective of this study was to determine the biochemical and thermal properties of the crude exo-polygalacturonase (from here on for simplicity polygalacturonase only) mostly considered among pectinases. This enzyme was produced by *Aspergillus sojae* ATCC 20235, not recognized so far. In our previous work (Gogus, Tari, Unluturk, Oncu, & Tokatlı, 2006; Tari, Gogus, & Tokatlı, 2007; Ustok, Tari, & Gogus, 2007), we reported that the organism exhibits a major potential for the production of this enzyme in submerged and solid state fermentation. Therefore, this paper will complement our previous studies.

In fact, the search for polygalacturonases with new industrial potential requires the discovery of new microbial strains and an understanding of the structure–stability relationship of this enzyme. The knowledge gained, will improve the potential and its effective usage in such diverse and broad areas. Furthermore, it will help to establish additional information required to maintain the desired level of enzyme activity over a long period of time and improve its stability. These are important parameters taken into account in the selection and design of enzymes (Gummadi & Panda, 2003). In this study characterization of the crude enzyme was considered, since the crude enzyme application can offer advantages in certain circumstances. For example, the collaborative action of different pectolytic enzymes in a crude enzyme sample preparation can increase the overall yield of the extraction or clarification process in the fruit juice industry. In fact a brief comparison of the crude enzyme with the purified enzyme, using three-phase partitioning (Dogan & Tari, 2008), is given at the end of this paper. It has been reported that a crude enzyme is more stable than a

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Nomenclature

E_d	inactivation energy (kJ mol^{-1})	T	absolute temperature (K)
ΔG^*	Gibb's free energy change (kJ mol^{-1})	V_{max}	maximum reaction velocity (μmol min^{-1})
h	Plank's constant (J s)	K_m	Michaelis constant (g l^{-1})
ΔH^*	enthalpy change (kJ mol^{-1})		
k_d	first-order deactivation rate constant (min^{-1})	*Greek symbol*	
R	universal gas constant (J mol^{-1} K^{-1})	κ	Boltzmann's constant (J K^{-1})
ΔS^*	entropy change (J mol^{-1} K^{-1})		
$t_{1/2}$	half-life time of enzyme (min)		

purified enzyme (Naidu & Panda, 2003). This observation was in agreement with our own results, highlighted in this paper.

2. Materials and methods

2.1. Microorganism and development of inoculum

Aspergillus sojae ATCC 20235 was purchased from Promochem Inc., an international distributor of ATCC (American Type of Culture Collection) in Europe. The propagation was done on yeast malt extract (YME) agar and incubated at 30 °C until sporulation. Stock cultures were prepared in 20% glycerol water and stored at −80 °C.

The spore suspensions used as inoculum were obtained on molasses agar slants, described in Tari et al. (2007).

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

2.2. Production of polygalacturonase

The production of polygalacturonase was done in shaken flasks (50 ml in 250 ml Erlenmeyer) containing glucose (25 g l^{-1}), peptone (2.5 g l^{-1}), disodium phosphate (3.2 g l^{-1}), monosodium phosphate (3.3 g l^{-1}) and maltrin (120 g l^{-1}). The fermentation was conducted at 350 rpm at 30 °C for 96 h. After this time, each flask was assayed for polygalacturonase enzyme activity. Enzyme activity was determined on supernatant obtained after the centrifugation of the broth at 6000 rpm for 15 min.

2.3. Enzyme assay

Polygalacturonase activity was defined and assayed according to the procedure given by Panda, Naidu, and Sinha (1999), using polygalacturonic acid as substrate at pH 6.6 and 26 °C.

2.4. Protein determination

The total protein contents of samples were determined according to the method reported by Lowry, Rosebrough, Farr, and Randall (1951); the protein standard used was bovine serum albumin.

2.5. SDS-PAGE profiling

SDS-PAGE was performed according to the procedure described by Laemmli (1970) using 10% resolving and 5% stacking gel. Protein bands were visualized using Coomassie brilliant blue R250 and methanol–water (containing 10% acetic acid) as staining and destaining agents, respectively. Fermentas SM0661 was used as the molecular weight marker, with the broad range of 10–200 kDa.

2.6. Effect of pH on activity and stability

The effect of pH on the activity of polygalacturonase was determined by assaying the enzyme activity at different pH values ranging from 3.0 to 7.0, using 0.1 M concentrations of the following buffer systems: citrate (pH 3.0), acetate (pH 4.0, 5.0) and phosphate (pH 6.0, 7.0). The relative activities were based on the ratio of the activity obtained at a certain pH to the maximum activity obtained at that range and expressed as a percentage. The pH stability of *A. sojae* polygalacturonase was investigated in the pH range 3.0–8.0, using 0.1 M citrate (pH 3.0), acetate (pH 4.0, 5.0) and phosphate (pH 6.0, 7.0, 8.0) buffer systems. Therefore, 2 ml of the crude enzyme were mixed with 2 ml of the buffer solutions indicated above and incubated at 30 °C for 2 h. After this period, aliquots of the mixtures were taken to measure the residual polygalacturonase activity (%) with respect to control, under standard assay conditions.

2.7. Effect of temperature on activity and stability

In order to determine the effect of temperature on the activity of polygalacturonase, the standard polygalacturonase assay procedure at different temperatures, ranging from 25 to 75 °C (25, 37, 45, 55, 65 and 75 °C), was performed. Prior to the addition of the enzyme, the substrate (0.24% (w/v) polygalacturonic acid) was pre-incubated at the respective temperature for 10 min. The relative activities as percentages, were expressed as the ratio of the polygalacturonase activity at a certain temperature to the maximum activity at the given temperature range. The thermostability of the crude 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 30 and 60 min.

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

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

$$t_{1/2} = \frac{\ln 2}{k_d}. \quad (1)$$

The temperature dependence of k_d was analyzed using the Arrhenius plot (Shuler & Kargi, 2002). The inactivation energy was calculated from the Arrhenius equation as

$$\ln(k_d) = \ln(k_0) - \left(\frac{E}{R}\right)\frac{1}{T}. \quad (2)$$

The values of E and k_0 were estimated from the slope and intercept of the plot of $\ln(k_d)$ versus $1/T$, respectively.

2.9. Estimation of thermodynamic parameters

The enthalpy of inactivation (ΔH^*) for each temperature was calculated according to Eq. (3)

$$\Delta H^* = E_d - RT. \quad (3)$$

The values for the Gibb's free energy (ΔG^*) of inactivation at different temperatures were calculated from the first-order constant of inactivation process by using Eq. (4)

$$\Delta G^* = -RT \ln\left(\frac{k_d h}{\kappa T}\right). \quad (4)$$

From Eqs. (3) and (4) the entropy of inactivation (ΔS^*) of polygalacturonase was calculated from Eq. (5)

$$\Delta S^* = \frac{(\Delta H^* - \Delta G^*)}{T}. \quad (5)$$

2.10. Determination of kinetic constant

A Lineweaver–Burk double reciprocal ($1/V$ versus $1/S$) plot (Shuler & Kargi, 2002) was used in order to determine the kinetic constants (V_{max} and K_m), where different concentrations of polygalacturonic acid were used as substrate (0.125–1 mg ml^{-1}) at pH 6.6

$$\frac{1}{v} = \frac{1}{V_m} + \frac{K_m}{V_m}\frac{1}{[S]}. \quad (6)$$

With a slope of K_m/V_m and intercept of $1/V_m$ (Eq. (6)), K_m and V_m values were estimated.

3. Results and discussion

3.1. Effect of pH on polygalacturonase activity and stability

As is seen from Fig. 1a, the enzyme was active over a broad pH range, displaying over 80% of its activity in the pH range 5.0–7.0. Optimum polygalacturonase activity was observed at pH 5. However, a further decrease from pH 5.0 to 3.0 decreased the polygalacturonase activity rapidly to 40% relative activity. These findings were in agreement with several studies where a pH optimum of 4.8 for polygalacturonase from *Aspergillus niger*, a pH optimum of 5.0 for *Aspergillus awamori* and a pH optimum of 4–5.5 for *Aspergillus japonicus* was reported (Jayani et al., 2005; Gummadi & Panda, 2003). It has been indicated that, among the polygalacturonases obtained from different microbial sources, most have an optimal pH range of 3.5–5.5 (Jayani et al., 2005). With an optimum pH of 5.0, *A. sojae* polygalacturonase could be applicable to fruit juice industries and wine making. It is well known that acidic pectinases mostly originate from fungal sources, especially from *A. niger* (Kashyap, Vohra, Chopra, & Tewari, 2001).

The effect of pH on stability (Fig. 1b) showed that the polygalacturonase enzyme was very stable at pH 5.0 and retained 60% and 70% of its activity at pH 3.0 and 7.0, respectively. The enzyme lost about 60% of its activity at pH 8.0. Therefore, polygalacturonase like the one characterized in this study could be a potential candidate for different applications in the industry requiring broader pH stability ranges.

3.2. Effect of temperature on activity and stability of polygalacturonase

The crude polygalacturonase was found to have an optimum temperature of 55 °C (Fig. 2a). The enzyme retained 75% of its activity at 65 and 75 °C. The polygalacturonase was active over a broad temperature range of 25–75 °C. As a close comparison, the optimum temperature of the commercial enzyme Rapidase C80 was determined at 55 °C as well (Ortega, de Diego, Perez-Mateos, & Busto, 2004). Similarly, in another study, the optimum temperature for *A. niger* polygalacturonase was reported to be 60 °C. This value was slightly higher than the partially purified polygalacturonase from *Sporotrichum thermophile Apinis*, having an optimum temperature of 55 °C (Jayani et al., 2005; Kashyap et al., 2001; Sakamoto, Bonnin, Quemener, & Thibault, 2002).

Thermostability of an enzyme is defined as the ability to resist thermal unfolding in the absence of substrates (Bhatti, Ashger,

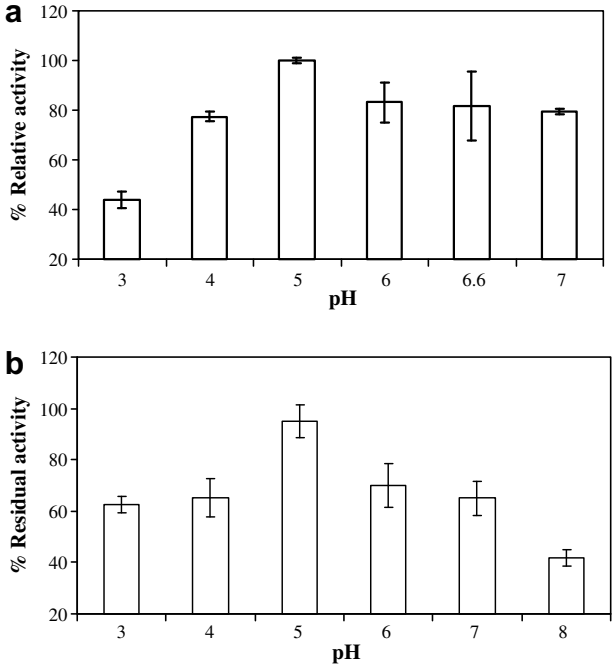

Fig. 1. Effect of pH on *A. sojae* PG (a) activity and (b) stability. Each experiment was carried out twice and the standard deviations of relative and residual polygalacturonase activity were 4.8% and 16.5%, respectively.

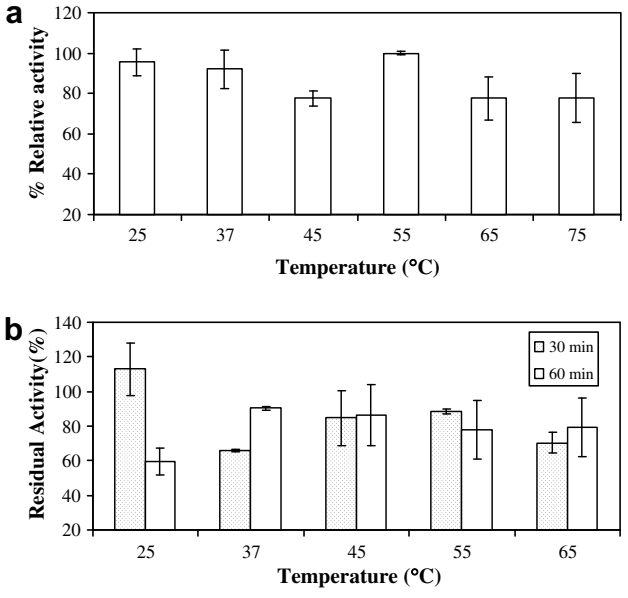

Fig. 2. Effect of temperature on *A. sojae* PG (a) activity and (b) stability. Each experiment was carried out twice and the standard deviations of relative and residual polygalacturonase activity were 11.3% and 15.2%, respectively.

Abbas, Nawas, & Sheikh, 2006). The thermostability of the polygalacturonase was measured using the residual activity of the enzyme after incubation at various temperatures ranging from 25 to 65 °C for 30 and 60 min (Fig. 2b). The enzyme was stable at 25 °C for 30 min of incubation, whereas 40% of its activity was lost after 1 h. This confirms that this enzyme favours higher temperatures as can be observed from the stability data at 37, 45, 55 and 65 °C. It has a residual activity ranging from 65% to 80% when incubated at these temperatures for 30 and 60 min. The thermostability of pectinases is an important parameter, especially in fruit juice extraction. Before the addition of pectinases, fruits are first cooked to release more juice. This releases most of the pectin into the juice, resulting in a thick and a cloudy appearance. Apples, stone fruits and berries are normally processed at 30–50 °C for about 15 to 90 min (Moyo et al., 2003). Therefore, *A. sojae* polygalacturonase has great potential to be used primarily in the fruit juice industry, because it is thermostable up to 65 °C for an hour. Since, after any application, the enzyme has to be inactivated, the estimation of thermal inactivation discussed in the next section has great importance.

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

The process whereby the secondary, tertiary or quaternary structure of a protein changes without breaking covalent bonds is defined as inactivation (Naidu & Panda, 2003). Inactivation rate constants (k_d) of polygalacturonase, presented in Table 1 at 75, 80, 82.5 and 85 °C, were calculated from the slope of the semilogarithmic plot of residual activity versus time. Likewise, the half-life values estimated using these constants and Eq. (1), are presented in the same Table. The half-life of polygalacturonase at 75 °C was 2.25, 4.97 and 3.53 times higher than the half-life values at temperatures of 80, 82.5 and 85 °C, respectively. This issue reveals the thermal stability of this enzyme at 75 °C and its easy inactivation at higher temperatures.

Inactivation energy of the crude polygalacturonase was determined as E_d = 152 kJ mol^{-1} from the slopes of the linear curve plotted by $1/T$ versus $\ln k_d$ using Eq. (2). This value was in close approximation to the values (167–293 kJ mol^{-1}) estimated for many microbial enzymes (Shuler & Kargi, 2002). The inactivation energy estimated for this enzyme was 1.05, 0.92 and 0.96 times higher than the commercial polygalacturonases Rapidase C80, Pectinase CCM and Pectinex 3XL, respectively (Ortega et al., 2004). These close approximations indicated that the crude enzyme can be considered as a potential candidate for various industrial applications.

3.4. Estimation of thermodynamic parameters during inactivation of polygalacturonase

The changes in enthalpy (ΔH^*) and entropy (ΔS^*) are calculated using transition state theory (Ortega et al., 2004) according to Eqs. (3) and (5) for the thermal inactivation of polygalacturonase (Table 2). A positive ΔH^* and ΔS^* were determined in the temperature ranges studied. With increase of temperature, a slight decrease in ΔH^* and a marked increase in ΔS^* were observed. This suggested thermal denaturation of the enzyme, possibly due to disruption of non-covalent linkages, including hydrophobic interactions (Bhatti et al., 2006; Georis et al., 2000). The opening up of the enzyme structure was in fact confirmed by the increase in the entropy or disorder of inactivation (Bhatti et al., 2006). The increase in ΔS^* also indicated an increase in number of protein molecules in transition activated stage, resulting in lower values of ΔG^*. Also, positive entropy values suggested that enzyme unfolding might be the rate determining step for the irreversible thermo-inactivation of polygalacturonase. Moreover, solvent and structural effects are reported to be the two major factors influencing the numerical values of ΔH^* and ΔS^*. Particularly, ΔS^* values are known to provide information regarding the degree of solvation and the degree of compactness of protein molecule (Dogan & Tari, 2008). Also the increase in the ΔH^* with respect to temperature increase reveals that the conformation of the enzyme was altered. The current results were in very close agreement with the results reported by Ortega et al. (2004) using commercial pectinases such as Rapidase C80, Pectinase CCM and Pectinex 3XL. In fact this is a positive outcome since we demonstrated that the polygalacturonase in the current study is very close in biochemical, as well as in thermal, characteristics, to commercial enzymes currently used.

Table 1
Kinetic parameters for thermal inactivation of polygalacturonase from *A. sojae*

T (°C)	k_d (min^{-1})	$t_{1/2}$ (min)
75	0.0032	216.60
80	0.0074	93.69
82.5	0.0159	43.59
85	0.0113	61.34

From slope of the semilogarithmic plot of residual activity versus time for each temperature, the inactivation rate constants (k_d) were calculated (R^2 = 0.94 for 85 °C, R^2 = 0.90 for 82.5 °C, R^2 = 0.96 for 80 °C and R^2 = 0.83 for 75 °C).

Table 2
Thermodynamic parameters of thermal inactivation of polygalacturonase from *A. sojae* at different temperatures

T (K)	ΔH^* (kJ mol^{-1})	ΔG^* (kJ mol^{-1})	ΔS^*(J mol^{-1} K^{-1})
348	149	102	134
353	149	99.9	139
355.5	149	97.7	144
358	149	98.7	140

3.5. Calculation of kinetic constants

The kinetic parameters of polygalacturonase, describing its affinity towards polygalacturonic acid (PGA) at pH 6.6 and 26 °C were obtained by a typical double reciprocal Lineweaver Burk plot (Eq. (6)). The apparent K_m value and V_m value for hydrolyzing PGA were V_m = 80 μmol min^{-1}, K_m = 0.424 g l^{-1}, respectively. The estimated K_m value was 1.7 and 1.87 times lower than the *A. niger* and *Sclerotinia sclerotiorum* polygalacturonases, respectively. This indicates that the current polygalacturonase has a higher affinity for PGA than the other two (Jayani et al., 2005). A similar K_m value (0.416 g l^{-1}) was reported in a study conducted using *Sporotrichum thermophile* polygalacturonase (Kaur, Kumar, & Satyanarayana, 2004). Another study with *A. niger* polygalacturonase indicated a V_m value of 154 μmol min^{-1} which is in correlation with our results (Singh & Rao, 2002).

3.6. SDS-PAGE profiling

SDS-PAGE profiling (Fig. 3) revealed that the crude enzyme was composed of three main fractions with molecular weights of 36, 53 and 68 kDa, respectively. The first two bands were observed after the purification step by means of three-phase partitioning as well (Gummadi & Panda, 2003). These bands could indicate the presence of isozymes, different proteins or impurities. The identifications remain a task to perform in future. Two different 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 *Penicillium frequentans,* respectively (Jayani et al., 2005). Therefore, the current enzyme will be a new example in the literature.

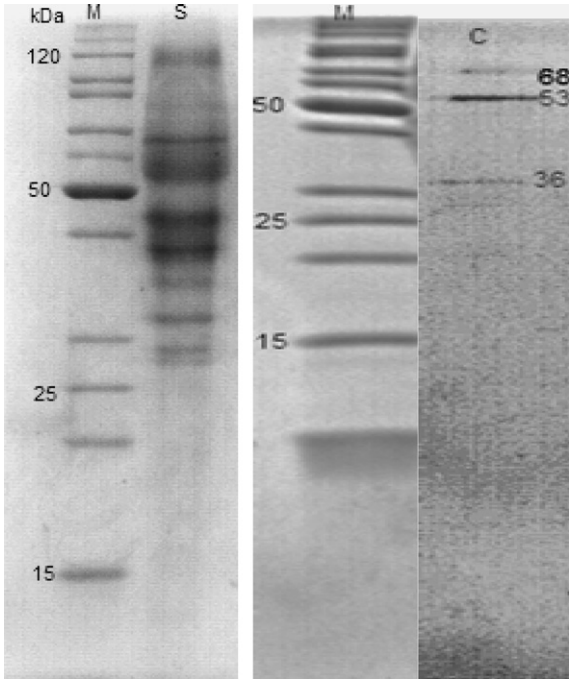

Fig. 3. SDS-PAGE of crude polygalacturonase (PG). Lane M, marker proteins; lane S, standard pectinases; lane C, crude extract. The amount of protein loaded in each sample lane was 20 μg.

3.7. Comparison of crude and purified polygalacturonase

Crude polygalacturonase was purified by a three-phase partitioning (TPP) procedure, a combination of ammonium sulphate and *tert*-butanol, to precipitate proteins from crude extracts as a single purification step (Dogan & Tari, 2008). In the comparison of the two enzymes (crude and purified enzyme using TPP), the optimum pH and temperatures were very close, with slight differences that could be related to the composition of the two enzyme systems used. It is also assumed that these results could be linked to the degree of purification applied. Perhaps, this procedure might not have been sufficient to eliminate some of the potential interfering substances affecting the activity. Furthermore, other proteins or impurities, as apparent in the SDS-PAGE profiling discussed above (Section 3.6), revealed this fact. However, the thermodynamic study revealed that the crude polygalacturonase was more stable than was the purified polygalacturonase. This observation was also reported by Naidu and Panda (2003), who explained this by the interactive effects among the three enzyme components, or proteins other than those components secreted by the organism and/or a combination of the two. Considering the kinetic parameters, the purified polygalacturonase showed a higher K_m value than the crude polygalacturonase. This could be a result of the presence of the other polygalacturonases that could not been purified by the three-phase partitioning method. A similar conclusion is also drawn from the V_m values, revealing a lower catalytic activity of the purified polygalacturonase. Finally, the biochemical and thermal characteristics of these two enzyme systems are clearly different. This signifies the importance of the application of crude versus purified enzymes in the industry.

As is known, pectinases have considerable industrial applications in several conventional processes, such as those for textiles, tea, coffee, oil extraction, fruit juice extraction and clarification. In fruit juice extraction, pectinases have been used in combination with enzymes such as cellulases, xylanases and arabinases to increase the pressing efficiency. Besides the food industry, pectinases have been used, together with cellulases, hemicellulases, amylases and lipases, in the textile industry (Jayani et al., 2005). Crude polygalacturonase extract can have great applications in the industry given that other pectinolytic enzymes and different enzymes can exist in the extract. These are already being used in the industry as a mixture in order to increase the process efficiency. Also, the high cost of enzyme purification procedures makes the crude extract preferable.

4. Conclusion

After biochemical and thermal characterization of the crude enzyme it seems that this enzyme could be a potential candidate for applications primarily in food, waste treatment centres, paper and textile industries. As a polygalacturonase from a strain, not previously considered for this purpose, it is a new example. With the promising results obtained, it may also be considered as an alternative to the commercial strain, *A. niger*. Discovering new enzymes with novel properties is a difficult task. Hence enzymes from new sources may have considerable economic values, which should not be ignored.

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