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Purification, Characterization, Kinetic Properties, and Thermal Behavior of Extracellular Polygalacturonase Produced by Filamentous Fungus *Tetracoccosporium* sp.

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Abstract

For the first time, a polygalacturonase from the culture broth of *Tetracoccosporium* sp. was isolated and incubated at 30°C in an orbital shaker at 160 rpm for 48 h. The enzyme was purified by ammonium sulfate precipitation and two-step ion-exchange chromatography and had an apparent molecular mass of 36 kDa, as shown by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Its optimum activity was at pH 4.3 and 40°C, and the K_m and V_{max} values of this enzyme (for polygalacturonic acid) were 3.23 mg/mL and 0.15 µmol/min, respectively. Ag^{+}, Co^{2+}, EDTA, Tween-20, Tween-80, and Triton X-100 stimulated polygalacturonase activity whereas Al^{3+}, Ba^{2+}, Ca^{2+}, Fe^{2+}, Fe^{3+}, Ni^{2+}, Mg^{2+}, Mn^{2+}, and SDS inhibited it. In addition, iodoacetamide and iodoacetic acid did not inhibit enzyme activity at a concentration of 1 m*M*, indicating that cysteine residues are not part of the catalytic site of polygalacturonase. We studied the kinetic properties and thermal inactivation of polygalacturonase. This enzyme exhibited a $t_{1/2}$ of 63 min at 60°C and its specific activity, turnover number, and catalytic efficiency were 6.17 U/mg, 113.64 min^{-1}, and 35.18 mL/(min·mg), respectively. The activation energy ($\Delta E^{\#}$) for heat inactivation was 5.341 kJ/mol, and the thermodynamic activation parameters $\Delta G^{\#}$, $\Delta H^{\#}$, and $\Delta S^{\#}$ were also calculated, revealing a potential application for the industry.

Index Entries: Polygalacturonase; *Tetracoccosporium* sp.; kinetic properties; thermal stability; thermodynamic activation parameters; purification.

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Introduction

Increasing energy demands have focused worldwide attention on the utilization of renewable resources, particularly agricultural and forest residues, the major components of which are cellulose, starch, lignin, xylan, and pectin. These materials have attracted considerable attention as an alternative feedstock and energy source, because they are available abundantly *(1)*. The effective catalytic properties of enzymes have already promoted their introduction into several industrial products and processes. Recent developments in biotechnology, particularly in areas such as protein engineering and directed evolution, have provided important tools for the efficient development of new enzymes. This has resulted in the development of enzymes with improved properties for established technical applications and in the production of new enzymes tailor-made for entirely new areas of application in which enzymes have not previously been used. Natural microorganisms have, over the years, been a great source of enzyme diversity *(2)*.

Saprophytic and plant pathogenic fungi and bacteria produce an array of enzymes capable of degrading the complex polysaccharides of the plant cell wall, including pectin, the main constituent of the middle lamella of higher plant cells. Pectin is a heteropolysaccharide consisting of a linear polymer of α (1→4) bond D-galacturonic acid residues or polygalacturonic acid, which is partially esterified. Pectin-degrading enzymes are used for the extraction and clarification of fruit juices, the extraction of oil, the preparation of cellulose fibers for linen, and the manufacture of jute and paper. Pectinases were some of the first enzymes to be used in homes *(3–6)*. The biotechnological potential of pectinolytic enzymes from microorganisms as likely biologic catalysts in a variety of industrial processes has drawn a great deal of attention from various researchers worldwide *(7)*. One of these pectin-degrading enzymes is polygalacturonase (poly [1,4-α-D-galacturonide] glycanohydrolase, EC 3.2.1.15). Polygalacturonase (hydrolyzes glycosidic linkages between galacturonic acid residues in polygalacturonan, a major fraction of plant pectins *(3)*.

In most industrial applications, fungal polygalacturonases prove to be very useful owing to the higher enzyme activity and their optimum activity at a lower pH range, which is suited to most fruit- and vegetable-processing applications. Because of the relatively low temperature stability of the fungal enzyme preparations, maceration needs to be carried out at a temperature not exceeding 45°C, which necessitates the incorporation of a pasteurization step to limit the growth of mesophilic microorganisms *(4)*. Furthermore, in apple juice manufacturing enzymic clarification could be carried out at either 15°C for 12 h or 50°C for 1 h. Many fungal polygalacturonases are thermolabile and become irreversibly inactivated at about 60°C, with a few exceptions, such as polygalacturonases from *Penicillium*, *Rhizopus*, and *Sclerotinia*. Optimal temperature for polygalacturonase activity was in the range of 30–50°C, and at greater than 50°C,

inactivation was notable after a short period of heating *(8)*. Polygalacturonases from a large number of fungi have been purified and characterized.

The production of pectinolytic enzymes has been widely reported in bacteria and filamentous fungi *(9)*. However, it could be of interest to find new sources of enzymes in order to display new specificities. The present investigation studied the production, purification, and characterization of polygalacturonase of *Tetracoccosporium* sp.; kinetic properties; and thermal inactivation. Based on our review of the literature, this is the first report on the production, purification, and characterization of polygalacturonase from *Tetracoccosporium* sp. The irreversible thermal inactivation of enzyme *(8)* made the thermodynamic approach impossible, and, therefore, a kinetic strategy was used to measure the stability. Residual activity was measured as a function of time, and kinetic rate constants were used to investigate the effect of the solvent on protein stability. Consequently, an Arrhenius plot was derived and thermodynamic parameters, such as free energy ($\Delta G^{\#}$), enthalpy ($\Delta H^{\#}$), and entropy ($\Delta S^{\#}$), were calculated.

Materials and Methods

Chemicals

Polygalacturonic acid was obtained from Fluka (Switzerland). α-D-Galacturonic acid and 3,5-dinitrosalicylic acid (DNS) were from Sigma (St. Louis, MO). Q-Sepharose and Mono-Q-Sepharose were provided by Pharmacia (Uppsala, Sweden). All other chemicals were obtained from Merck (Darmstadt, Germany) and were reagent grade.

Fungal Strain and Growth Conditions

Saprotrophic hyphomycetes isolated from agricultural and vegetable wastes were obtained from the culture collection of Applied Microbiology Laboratory at Alzahra University. All morphologic contrasting colonies were purified by streaking *(4)*. The highly pectinolytic fungal strain was tentatively identified as *Tetracoccosporium* sp. according to the morphologic characterization *(10)*.

Preparation of Spore Suspension

Spores from sabouraud dextrose agar (SDA) slants were washed in 5 mL of 0.01% Tween-80 solutions. The spore suspension was adjusted to a final concentration in the culture medium of 10^6 spores/mL *(11,12)*.

Culture Medium and Conditions

Sterilization was carried out at 121°C and 15 psi for 20 min. The initial pH in all cases was 5.6. The medium contained the following: 5 g/L of polygalacturonic acid, 3 g/L of $(NH_4)_2SO_4$, 10 g/L of KH_2PO_4, 2 g/L of $MgSO_4 \cdot H_2O$, 0.7 mg/L of $Na_2B_4O_7 \cdot 10H_2O$, 0.5 mg/L of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 10 mg/L of $Fe_2(SO_4)_3 \cdot 6H_2O$, 0.3 mg/L of $CuSO_4 \cdot 5H_2O$, 0.11 mg/L of

$MnSO_4 \cdot H_2O$, 17.6 mg/L of $ZnSO_4 \cdot 7H_2O$, and 100 mg/L of streptomycin. All cultures were incubated at 30°C in an orbital shaker at 160 rpm for 48 h. Then samples were filtered through Whatman No. 4 filter paper, and the filtrate was assayed for enzyme activity.

Enzyme Catalytic Assay and Determination of Protein

Polygalacturonase activity was assayed by quantifying reducing groups expressed as galacturonic acid units that had been liberated during the incubation of 200 µL of 1% (w/v) polygalacturonic acid in 20 m*M* sodium acetate buffer, pH 4.3, with 200 µL of suitably diluted enzyme (0.047 mg/mL) at 25°C for 3 min by DNS method *(13)*. One unit of polygalacturonase was defined as the amount of enzyme required to release 1 µmol of galacturonic acid (as a standard) from the polygalacturonic acid/min.

Protein content was determined with Coomassie blue reagent using bovine serum albumin as a standard *(14)*.

Purification of Polygalacturonase

One thousand milliliters of the cell-free supernatant was saturated with ammonium sulfate to 90% saturation. The saturated solution was left overnight at 4°C, centrifuged at 10,000g for 20 min at 4°C, dissolved in a minimal amount of 10 m*M* sodium acetate buffer (pH 5.75), and dialyzed against the same buffer for 24 h at 4°C. Proteins were fractionated on a fast performance liquid chromatography system (Pharmacia) equipped with an anion-exchange chromatography column (Q-Sepharose fast flow, 1×10 cm; Pharmacia) equilibrated with buffer A (20 m*M* acetate buffer, pH 5.75). The column was eluted with a 10-column vol linear gradient of buffer B (20 m*M* acetate buffer, pH 5.75; 1 *M* NaCl) at a flow rate of 1 mL/min and fractions were collected. The elution was monitored by an ultraviolet detector. Polygalacturonase-containing fractions (unbound fractions) were pooled and then dialyzed with phosphate buffer (pH 6.1) before being fractionated by a Mono-Q-Sepharose column (0.5×5 cm). The column was equilibrated with buffer A (20 m*M* phosphate buffer, pH 6.1) and eluted with a 10–column vol gradient of buffer B (20 m*M* phosphate buffer, pH 6.1; 1 *M* NaCl) at a flow rate of 0.5 mL/min, and the polygalacturonase-containing fractions (unbound fractions) were mixed.

Gel Electrophoresis

The purity and molecular masses of polygalacturonase were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a slab gel prepared with 12% (resolving gel) and 5% (stacking gel) acrylamide *(15)*. The protein samples were denatured by heating them at 100°C with the sample buffer for 5 min before loading. The relative molecular mass of the protein was estimated using standard molecular weight (premixed protein molecular weight marker; Roche). The protein bands were stained by silver nitrate *(16)*.

Determination of Kinetic Parameters

Polygalacturonic acid was solubilized in 20 m*M* acetate buffer, pH 4.0, and incubated at room temperature. The tested substrate concentrations were in the range of 0.01–4% (w/v) of polygalacturonic acid. After adding the polygalacturonase, aliquots of the digests were withdrawn at fixed time intervals and the release of the reducing end was monitored by the Nelson method *(14)*. Michaelis parameters K_m and V_{max} were calculated from Lineweaver-Burk plots $1/V_0$ vs $1/[S]$, in which $[S]$ is the concentration of the substrate and V_0 is the initial rate of the hydrolysis. The catalytic efficiency was calculated as the ratio V_{max}/K_m *(8)*.

Effect of pH and Temperature on Polygalacturonase Activity

Optimal temperature and pH were estimated using a temperature range of 10–90°C and a pH range of 2.0–10.0. pH stability was studied by preincubating the enzyme at room temperature for 90 min at various pH values in 10 m*M* mix buffer (glycine:HCl buffer, pH 2.2–3.4; acetate buffer, pH 3.6–5.4; phosphate buffer, pH 5.8–8.0; glycine:NaOH buffer, pH 8.4–10.0) All the enzyme activities were assayed using polygalacturonic acid (0.5%) as the substrate.

Effect of Metal Ions, Metal Chelators, and Chemical Compounds on Polygalacturonase Activity

Metal ions, metal chelators, and chemical compounds (Ag^+, Al^{3+}, Ba^{2+}, Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, K^+, Ni^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, EDTA, iodoacetamide, iodoacetic acid, Tween-20, Tween-80, Triton X-100, and SDS) were added to the substrate buffer (0.5% polygalacturonic acid in 20 m*M* acetate buffer, pH 4.3), and polygalacturonase activity was measured as previously described.

Irreversible Thermoinactivation of Enzyme

Thermal stability was investigated by measuring the residual activity of the enzyme after 10 and 20 min of incubation at different temperatures. In another method, samples were thermally treated at 50–80°C for up to 60 min. The residual activity was calculated as follows:

$$\text{Residual polygalacturonase activity, \%} = 100(A_t/A_0)$$

in which A_t is the activity at time t (s), and A_0 is the activity at time $t = 0$ s.

Inactivation enzymes can often be described by a first-order kinetic model; that is, the enzyme activity decreases log linearly as a function of time, as described by the following equation:

$$\ln(A_t/A_0) = -kt$$

in which A_t is the enzyme activity at time t, A_0 is the initial enzyme activity, t is the treatment time, and k is the first-order inactivation rate constant.

Calculation of Thermodynamic Parameters

The temperature dependence of the rate constant for the inactivation was analyzed according to Arrhenius plot. The activation energy ($\Delta E^{\#}$) was obtained from the slope of the Arrhenius plot (regression of logarithm of reaction rate constant vs reciprocal of absolute temperature). The activation enthalpy ($\Delta H^{\#}$) for each temperature was calculated as follows:

$$\Delta H^{\#} = \Delta E^{\#} - RT$$

in which R is the universal gas constant and T is the absolute temperature. The values for free energy of the inactivation ($\Delta G^{\#}$) at different temperatures were calculated from the first-order rate constant of the inactivation process by the following equation:

$$\Delta G^{\#} = -RT \ln(kh/K_B T)$$

in which h (=6.62×10^{-34} J/s) is the Planck constant, and K_B (=1.3806×10^{-23} J/K) is the Boltzmann constant. From the two previous equations, the activation entropy ($\Delta S^{\#}$) for polygalacturonase heat inactivation was calculated as follows:

$$\Delta S^{\#} = (\Delta H^{\#} - \Delta G^{\#})/T$$

Results and Discussion

Purification of Polygalacturonase

Polygalacturonase production by *Tetracoccosporium* sp. was high after 2 d of cultivation. Enzyme production was increased by stirring at 160 rpm as compared to the static conditions. The polygalacturonase was purified from the culture broth. The supernatant was first concentrated by ammonium sulfate precipitation. The polygalacturonase was then separated from other proteins by two-step anion-exchange chromatography: Q-Sepharose and Mono Q-Sepharose. Both methods revealed that polygalacturonase activity was found in the unbound fraction (Fig. 1A,B). Table 1 summarizes the purification steps. SDS-PAGE showed one major band with a molecular mass of 36 kDa (Fig. 1C).

Effect of pH and Temperature on Polygalacturonase Activity

The polygalacturonase of *Tetracoccosporium* sp. was quite active over a wide pH range (3.0–8.0), with its optimum at 4.3 (Fig. 2). This finding is interesting and important for the beverage industry because of the acidic pH of juices. A similar pH range for the activity of the enzyme was reported in *Sporotrichum thermophile (1)*, *Streptomyces* sp. QG-11-3 *(17)*, *Verticillum albo-atrum (18)*, *Aspergillus niger (19)*, *Lentinus edodes (20)*, *Rhizopus* spp. *(21)*, and Pectinol A1 and Röhapect D5S from Röhm *(22)*.

The enzyme was active in the temperature range of 20–70°C, with optimal activity at 40°C (Fig. 3A). A comparable thermostability was found

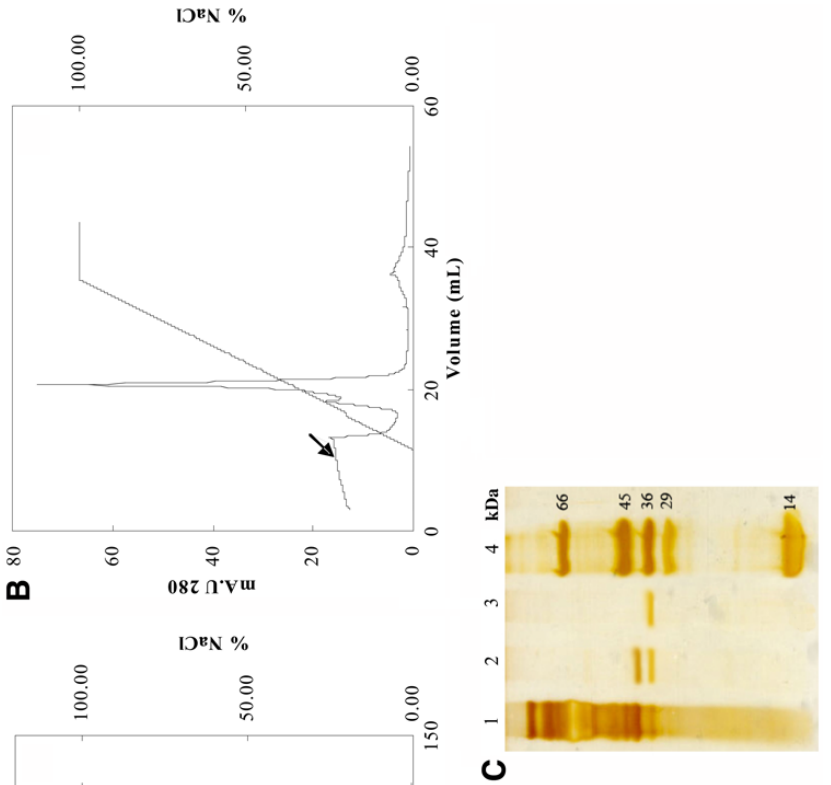

Fig. 1. Elution profiles of *Tetracoccosporium* sp. polygalacturonase on **(A)** Q-Sepharose and **(B)** Mono-Q-Sepharose. Arrows indicate the active peaks. Silver staining SDS-PAGE illustrates different steps of the polygalacturonase purification. **(C)** Lane 1, crude enzyme after precipitation with $(NH_4)_2SO_4$; lane 2, after ion-exchange chromatography using Q-Sepharose column; lane 3, after Mono-Q-Sepharose column (the purified enzyme); lane 4, molecular size markers.

Table 1
Procedure for Purification

Purification step	Volume (mL)	Activity (U)	Specific activity (U/mg)	Protein (mg/mL)	Fold purification	Recovery (%)
Ammonium sulfate	21	0.6	1.39	0.42	1	100
Q-Sepharose	19	0.4	5.97	0.07	4.29	69
Mono Q-Sepharose	12	0.3	26.36	0.01	18.96	50

for pectinases of *Streptomyces* sp. QG-11-3 *(17)* and *Clostridium thermosaccharolyticum (23)*, whereas higher temperature optima of 45 and 50°C were reported for pectinases from *Sclerotinia sclerotiorum (24)* and *Saccharomyces cerevisae (25)*, respectively.

Effect of Metal Ions, Metal Chelators, and Chemical Compounds on Polygalacturonase Activity

The activity of polygalacturonase was stimulated by Ag^{+}, Co^{2+}, EDTA, Tween-20, Tween-80, and Triton X-100, whereas Al^{3+}, Ba^{2+}, Ca^{2+}, Fe^{2+}, Fe^{3+}, Ni^{2+}, Mg^{2+}, Mn^{2+}, and SDS inhibited activity. Iodoacetamide and iodoacetic acid did not inhibit the enzyme activity at a 1 m*M* concentration, which indicated that cysteine residues are not part of the catalytic site of this enzyme. Surface-active detergent such as Tweens (20 and 80) and Triton X-100 stimulated polygalacturonase activity. Perhaps this is owing to the fact that the surface-active detergent might have increased the turnover number of polygalacturonase by increasing the contact frequency between the active site of the enzyme and the substrate by lowering the surface tension of the aqueous medium (Table 2) *(26)*. The pectinolytic enzymes of *S. thermophile* were stimulated by Fe^{2+}, Mn^{2+}, Ca^{2+}, and Cu^{2+} at 1 m*M* and inhibited by Mg^{2+} strongly *(1)*. The pectinolytic enzymes of *S. sclerotinium* were inhibited by Hg^{2+}, Zn^{2+}, Cu^{2+} and stimulated by Mn^{2+} and Co^{2+} *(24)*. The polygalacturonase of *Bacillus* MG-cp-2 was stimulated by Ca^{2+} *(26)*. The enzyme activity of *S. thermophile* was slightly inhibited by Tween-80 and Triton X-100, and a complete loss of *S. thermophile* enzyme activity was observed in the presence of SDS *(1)*.

Kinetic Parameters of Polygalacturonase

The K_m (for polygalacturonic acid) and V_{max} values were calculated from a Lineweaver-Burk plot (Fig. 4). The graph was linear with a correlation coefficient (R^2) of 0.9699. The values of K_m and V_{max} were 3.23 mg/mL and 0.15 ?mol/min, respectively. In comparison with the K_m values of the polygalacturonase of *Neurospora crassa* (5.0 mg/mL) and *Paenibacillus amylolyticus* (4.6 mg/mL) *(27,28)*, the polygalacturonase of *Tetracoccosporium* sp. exhibited a high affinity for polygalacturonic acid. Table 3 gives the kinetic parameters of the enzyme. k_{cat}, or turnover number, was calcu-

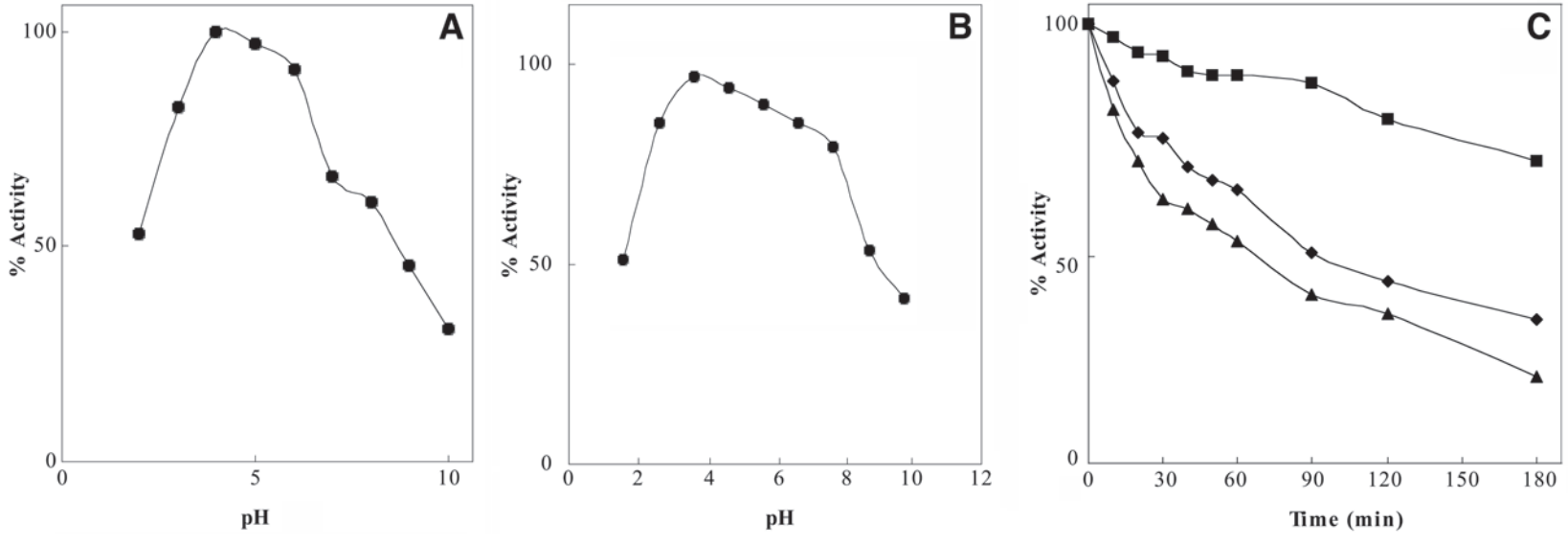

Fig. 2. **(A)** Effect of pH on enzyme activity at 25°C. A mixture of glycine, acetate, and phosphate buffers at a concentration of 10 m*M* was used. **(B)** pH stability of purified enzyme at pH 2.0–10.0 for 90 min at 25°C. **(C)** Remaining activity of purified enzyme at pH 2.0 (◆), 7.0 (■), and 10.0 (▲) at 25°C. For further details, see Materials and Methods.

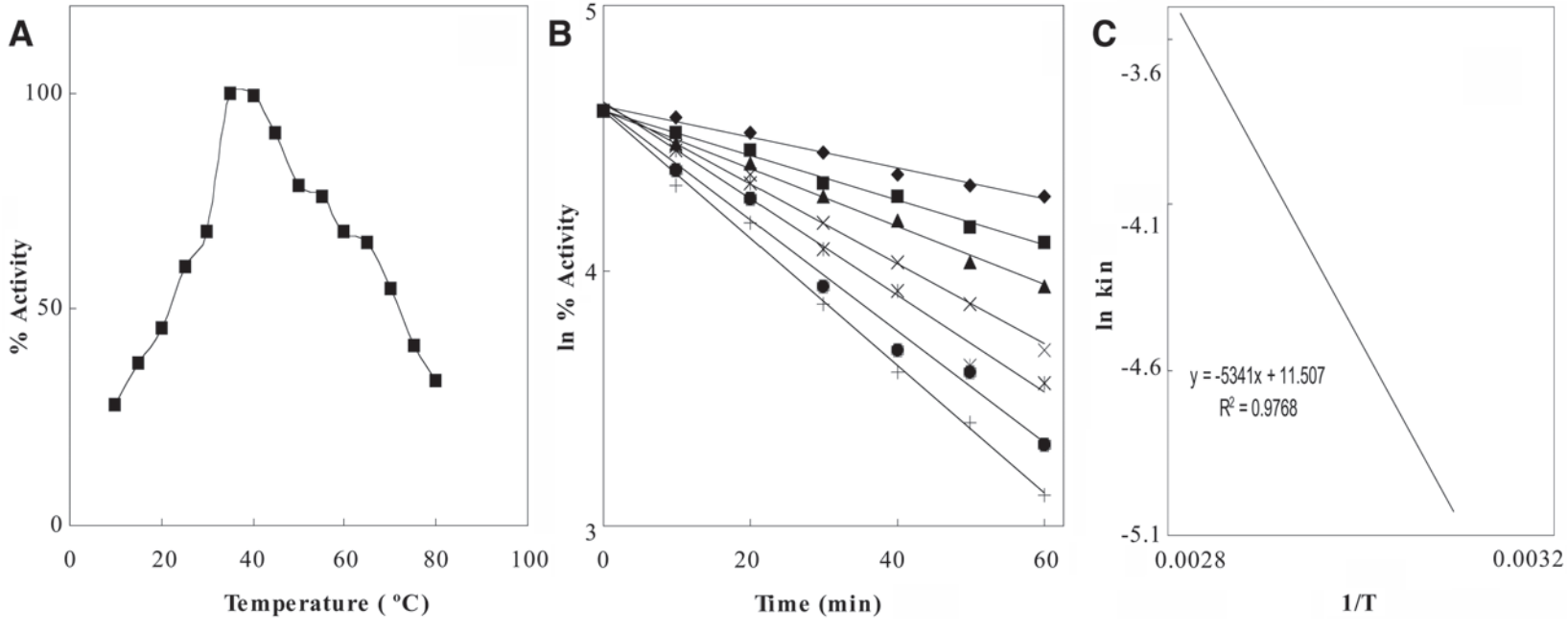

Fig. 3. **(A)** Temperature profile of purified enzyme in 20 m*M* acetate buffer, pH 4.3. **(B)** Thermal inactivation kinetics of polygalacturonase activity at 50–80°C. The assays were incubated at various temperatures ([◆] 50°C; [■] 55°C; [▲] 60°C; [×] 65°C; [*] 70°C; [●] 75°C; [+] 80°C) prior to the addition of substrate, followed by cooling in an ice bath. The activity at zero time was taken as 100% activity. **(C)** Arrhenius plot for heat inactivation of polygalacturonase. The activation energy was 5.341 kJ/mol.

Table 2
Effect of Metal Ions (1 m*M*), Metal Chelators, and Chemical Compounds on Polygalacturonase Activity

Metal ion/ chemical compound	Residual activity of polygalacturonase
Control	100
Ag^{+}	112
Al^{3+}	77
Ba^{2+}	79
Ca^{2+}	81
Co^{2+}	108
Cu^{2+}	100
Fe^{2+}	85
Fe^{3+}	77
K^{+}	100
Ni^{2+}	79
Mg^{2+}	85
Mn^{2+}	77
Zn^{2+}	97
EDTA (1 m*M*)	104
Iodoacetamide (1 m*M*)	110
Iodoacetic acid (1 m*M*)	108
Tween-20 (0.1% [v/v])	109
Tween-80 (0.1% [v/v])	113
Triton X-100 (0.1% [v/v])	102
SDS (0.1% [w/v])	87

lated as $V_{max}/[E]$. Catalytic efficiency was calculated as k_{cat}/K_m. The catalytic efficiency value therefore provides a useful model for selecting the most efficient enzyme for an industrial process using affixed initial substrate concentration *(29)*. In comparison with other polygalacturonases used in the clarification of fruit juice such as Pectinase CCM (12.8 mL/[min·mg]) and Pectinex 3XL (12.8 mL/[min·mg]) (8), *Tetracoccosporium* sp. polygalacturonase (35.2 mL/[min·mg]) shows a remarkable efficiency.

Stability Studies

The use of enzymes in industrial processes may require that reactions be conducted at high temperatures in order to improve productivity and reduce microbial contamination. Consequently, thermostable enzymes have been the subject of numerous studies involving the elucidation of thermal deactivation mechanisms and the development of strategies for stability enhancement *(30)*. Figure 3B shows the residual polygalacturonase activity vs heating time. Study of the thermal inactivation curve of the polygalacturonase from *Tetracoccosporium* sp. could be relevant to industrial applications. Thus, when the process was carried out at 60°C the half-life was 63.01 min (Fig. 3B). The half-life at 60°C for Pectinase CCM was

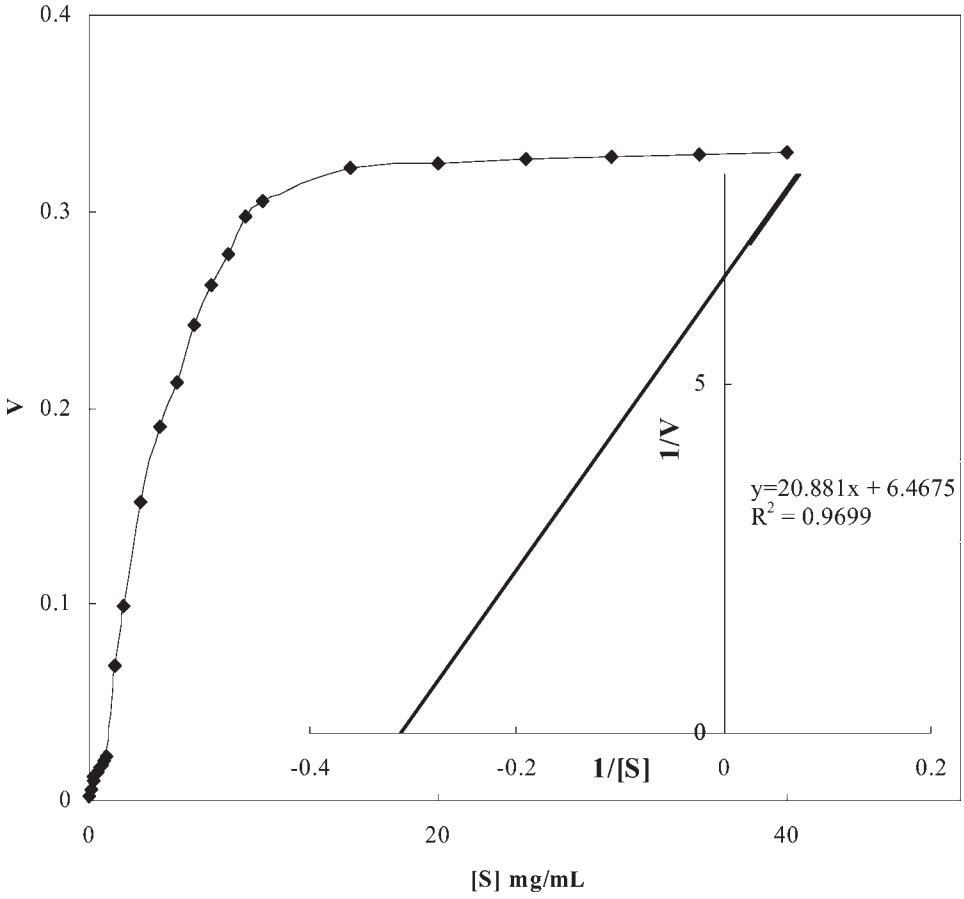

Fig. 4. Michaelis-Menten curve of polygalacturonase activity. (*Inset*) Lineweaver-Burk plot relating *Tetracoccosporium* sp. polygalacturonase reaction velocity to polygalacturonic acid concentration.

Table 3
Steady-State Kinetic Parameters of Polygalacturonase

Substrate	K_m (mg/mL)	V_{max} (U)	k_{cat} (1/min)	k_{cat}/K_m (mL/[min·mg])
Polygalacturonic acid	3.23	0.15	113.64	35.2

61.78 min and for Rapidase C80 was 7.80 min *(8)*. Devi and Rao *(31)* also showed that at 46°C purified polygalacturonase from *Aspergillus carbonarius* had a half-life of 8 min. Therefore, the polygalacturonase assayed in the present work has a remarkable heat-tolerance, which makes it very attractive for industrial applications. The inactivation rate constant (*k*) was determined from the slope of such plots (Fig. 3B). The estimated rate constant for

Table 4
Thermodynamic Parameter Values
of Thermal Inactivation of Polygalacturonase

Enzyme	$\Delta E^{\#}$ (kJ/mol)	$\Delta H^{\#}$ (kJ/mol)	$\Delta G^{\#}$ (kJ/mol)	$\Delta S^{\#}$ (J/mol·K)
Polygalacturonase	5.341	25.72	94.35	0.2

the inactivation of polygalacturonase values for the enzyme half-life ($t_{1/2}$) in minutes is calculated as follows:

$$t_{1/2} = \ln(2)/k \ (\text{min}^{-1})$$

Temperature Dependence of Polygalacturonase Inactivation

There is little published information concerning transition-state parameters for polygalacturonase heat inactivation. Agblor et al. *(32)*, Devi and Rao *(31)*, and Naidu and Panda *(33)* reported estimates of $\Delta E^{\#}$, $\Delta H^{\#}$, $\Delta S^{\#}$, and $\Delta G^{\#}$ for polygalacturonase from *Lygus* spp., *A. carbonarius*, and *A. niger*, respectively. The activation energy ($\Delta E^{\#}$) of polygalacturonase inactivation was determined using an Arrhenius model and in *Tetracoccosporium* sp. was 5.341 kJ/mol (Fig. 3C). Similar values have been reported for the polygalacturonase from *Rhizopus oryzae* CJ-2112 (8.56 kJ/mol) *(34)*; Rapidase C80, Pectinase CCM, and Pectinex 3XL (26.5, 45.6, and 4.20 kJ/mol, respectively) *(8)*; and *Rhizopus* spp. (27.2 kJ/mol) *(35)*. Table 4 gives values of the activation parameters $\Delta E^{\#}$, $\Delta H^{\#}$, $\Delta G^{\#}$, and $\Delta S^{\#}$.

At moderated temperatures, the rate-limiting step for the irreversible heat inactivation of enzymes is the formation of an unfolded enzyme (**U**) state, describing irreversible inactivation as a two-stage reaction *(36–40)*:

$$\mathbf{N} \leftrightarrow \mathbf{U} \rightarrow \mathbf{I} \text{ (and } \mathbf{N} \rightarrow \mathbf{I} \text{ overall)}$$

in which **N** is the native conformation for polygalacturonase, **U** is the heat-unfolded enzyme, and **I** is the irreversible inactivated polygalacturonase. Thermodynamic parameters are associated with the formation of a transition state (**Tn***) according to the following equation:

$$\mathbf{N} \leftrightarrow \mathbf{Tn}^* \rightarrow \mathbf{U}$$

The $\Delta G^{\#}$ value (94.35 kJ/mol) is of the order of magnitude usually expected for protein denaturation *(36)*. Similar values have been reported for Rapidase C80, Pectinase CCM, Pectinex 3XL, and pectolytic enzymes from *A. niger* (usually from 88 to 105 kJ/mol) *(8,33)*. The large activation enthalpy value is also characteristic of the protein denaturation reaction *(37–39)*. Therefore, the unfolding of the enzyme may be the rate-determining step for the irreversible thermoinactivation of the polygalacturonase under the assayed conditions. The $\Delta H^{\#}$ value for the polygalacturonase from *Tetracoccosporium* sp. was 25.72 kJ/mol. Similar values have been reported for Rapidase C80, Pectinase CCM, Pectinex 3XL, and pectolytic

enzymes from *A. niger* (from 39.92 to 166 kJ/mol) *(8,33)*. $\Delta H^{\#}$ and $\Delta S^{\#}$ are, respectively, the heat and the entropy change for the **N** → **Tn*** reaction. These parameters provide a measure of the number of broken noncovalent bonds and the net enzyme/solvent disorder change associated with the **N** → **Tn*** transition, respectively. The positive $\Delta S^{\#}$ value (0.2 J/mol) also suggests that the unfolding of the enzyme is the rate-determining step for the irreversible thermoinactivation of the native polygalacturonase (at 60°C). There is an increase in the entropy during the conversion of the **N** state to the **Tn*** state. A positive $\Delta S^{\#}$ value for Pectinex 3XL has also been previously reported *(8)*. However, the negative $\Delta S^{\#}$ values may be accounted for by possible aggregation of the partially unfolded enzyme molecules *(41)*, which predominate during the exposure of protein to high temperatures *(42)*. Negative $\Delta S^{\#}$ values have been reported for pectolytic enzymes from *A. niger (33)*.

Conclusion

A new technology based on total liquefaction may be developed by using pectinase, hemicellulase, and cellulase in combination for obtaining a clarified and depectinized juice in a single step. The addition of exogenous enzymes also allows more specific degradation, which is necessary to give a smooth texture to juice and is usually not found by heating, while also preserving the color and vitamins of the juice *(43)*.

Because of these characteristics, the polygalacturonase from *Tetracoccosporium* sp. could be widely used in clarification in the beverage industry and enhance the extraction of fruit juice with increased levels of reducing sugars. In the present study, the polygalacturonase of *Tetracoccosporium* sp. was biochemically characterized. A novel polygalacturonase was produced from a *Tetracoccosporium* sp. isolated from the environment. This polygalacturonase not only was active and stable under acidic conditions but also exhibited a broad range of thermostability at temperatures up to 80°C, as well as tolerance to metal ions and surface-active agents. This example is just one of the many ways commercial enzymes touch our lives. They are tools of nature that help provide everyday products in an environmentally conscious manner. The development of enzyme products often relies on screening a large number of organisms for an enzyme activity with a specific set of biochemical and biophysical characteristics that suits the targeted population. By combining enzyme screening with modern techniques of protein engineering, directed evolution, and metagenome approaches, new and novel biocatalysts with improved performance under specific applications and conditions can be generated.

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