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Purification and Characterization of Two Extracellular Xylanases from *Penicillium sclerotiorum*: A Novel Acidophilic Xylanase

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Abstract Two xylanases from the crude culture filtrate of *Penicillium sclerotiorum* were purified to homogeneity by a rapid and efficient procedure, using ion-exchange and molecular exclusion chromatography. Molecular masses estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis were 23.9 and 33.1 kDa for xylanase I and II, respectively. The native enzymes' molecular masses of 23.8 and 30.8 kDa were estimated for xylanase I and II, respectively, by molecular exclusion chromatography. Both enzymes are glycoproteins with optimum temperature and pH of 50 °C and pH 2.5 for xylanase I and 55 °C and pH 4.5 for xylanase II. The reducing agents β-mercaptoethanol and dithio-treitol enhanced xylanase activities, while the ions Hg^{2+} and Cu^{2+} as well the detergent SDS were strong inhibitors of both enzymes, but xylanase II was stimulated when incubated with Mn^{2+}. The K_m value of xylanase I for birchwood xylan and for oat spelt xylan were 6.5 and 2.6 mg mL^{-1}, respectively, whereas the K_m values of xylanase II for these substrates were 26.61 and 23.45 mg mL^{-1}. The hydrolysis of oat spelt xylan by xylanase I released xylobiose and larger xylooligosaccharides while xylooligosaccharides with a decreasing polymerization degree up to xylotriose were observed by the action of xylanase II. The present study is among the first works to examine and describe an extracellular, highly acidophilic xylanase, with an unusual optimum pH at 2.5. Previously, only one work described a xylanase with optimum pH 2.0. This novel xylanase showed interesting characteristics for biotechnological process such as feed and food industries.

Keywords *Penicillium sclerotiorum* · Xylanase · Enzyme purification · Enzyme characterization

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Introduction

β-1,4-Xylan is a major structural polysaccharide of plant cell walls being the second most prevalent in nature after cellulose. It is a heterogeneous polymer constituted primarily by a linear β-(1,4)-D-xylose backbone, which is partially acetylated and substituted in different degrees by a variety of side chains, mainly single α-D-glucuronosyl and α-L-arabinosyl units. Due to its structural complexity, several hydrolases are required for its complete degradation. The key enzyme in this process is endo-β-(1,4)-xylanase (EC 3.2.1.8), which cleaves the internal β-(1→4) bonds in the xylan backbone at non-modified residues, yielding different chain-length-substituted xylooligosaccharides [1–3].

Interest in xylanolytic enzymes has increased in recent years due to their potential application in biotechnology. Xylanases have been studied by their importance in several industrial processes, such as bioconversion of lignocellulosic materials into fermentative products, improvement of digestibility of animal feedstock, clarification of juices, and facilitation of the release of lignin from the pulp, thereby reducing the chlorine amount required for bleaching in pulp and paper industry [4–6]. In addition, they can be effectively used with cellulases to hydrolyze the lignocellulosic biomass generated which can be converted in bioethanol and xylitol [4, 7]. Xylanases are also employed for degumming of fibers, such as flax, hemp, jute, and ramie, and in baking to increase elasticity and strength of the dough leading to an improvement of loaf volumes and texture of bread [4, 8].

Xylanases are produced by a variety of microorganisms, including filamentous fungi and bacteria, and their enzyme systems have been most widely studied [3, 9]. In many of these microorganisms, multiple forms of xylanases have been observed. These enzymes may have diverse physico-chemical properties, structures, specific activities, and yields, increasing the efficiency and extent of xylan hydrolysis. Fungal xylanases are more interesting from an industrial point of view because their extracellular activities are much higher than those from yeasts and bacteria [9, 10]. Species of Aspergilli, Penicillia, and Tricodermii are examples of microorganisms which can produce xylanolytic isoenzymes. The Penicillia are mostly saprophytic in nature and numerous species are of particular value for humanity [11], many of them constituting a rich source of enzymes for xylan biodegradation [12].

Recently, we reported the best conditions for the xylanase production by a *Penicillium sclerotiorum* strain, isolated as a good xylanase producer [13]. In this paper, we described the purification and some properties of two extracellular xylanases produced by this fungus under optimized culture conditions. The acidophilic and elevated pH stability of one purified xylanase in this work has potential applications in feed and food industries.

Materials and Methods

Organism and Growth

P. sclerotiorum used in the present work is available in the Culture Collection of Environmental Studies Center—CEA/UNESP, SP, Brazil. Conidia were obtained from cultures in Vogel solid medium [14] containing 1.5% (*w*/*v*) glucose and 1.5% (*m*/*v*) agar at 25 °C for 7 days. Liquid cultures were prepared in the same medium with 1% (*w*/*v*) oat spelt xylan as carbon source and pH was adjusted to 6.5. Erlenmeyer flasks (125 mL) containing 25 mL of medium were inoculated with 1.0 mL of spore suspension containing 5×10^7 spores/mL and incubated at 30 °C for 5 days in stationary condition. The mycelium was removed by vacuum filtration and the crude culture filtrate was used as a source of extracellular proteins.

Enzyme Assay

Crude and purified xylanase activities were determined at 50 °C using 1.0% (*w*/*v*) birchwood xylan (Sigma, St. Louis, MO, USA) in McIlvaine buffer pH 4.5. This buffer is prepared from a mixture of 0.1 M citric acid and 0.2 M sodium monohydrogen phosphate. After 5 and 10 min of incubation, the reaction was interrupted by addition of 3,5-dinitrosalicylic acid and the reducing sugars released were quantified [15], using xylose as standard. One unit of enzyme activity was defined as the amount of enzyme capable of releasing 1 μmol of reducing sugar per minute, under assay conditions. Specific activity was expressed as unit per milligram of protein. All enzymatic assays were developed in triplicate and the results are presented through mean values.

Protein Determination

Total protein was determined by Lowry's method [16], using bovine serum albumin as standard.

Xylanase Purification Ion-Exchange Chromatography on DEAE-Sephadex A-50

The crude filtrate of the fungal culture (200 mL) was dialyzed using a 12-kDa cellulosic membrane against 8 L of 0.05 M Tris–HCl buffer pH 9.0 for 8 h. The dialyzed crude enzyme was chromatographed on DEAE-Sephadex A-50 column (2.8×17.8 cm) equilibrated with the same buffer. Bounded proteins were eluted by a 0.0–0.5 M NaCl linear gradient, at a flow rate of 60 mL/h and 3.0-mL fractions were collected. Absorbance at 280 nm was measured and xylanase activity was determined. The fractions with significant xylanase activity were pooled and the samples were subjected to electrophoresis. All the purification steps were carried out at 4 °C.

Molecular Exclusion Chromatography

The samples corresponding to the retained and non-retained fractions from ion-exchange column were further dialyzed against 8 L of 0.05 M ammonium acetate buffer pH 6.8 for 8 h with four changes, and then lyophilized and re-suspended in a small volume of this buffer. The samples were chromatographed on Sephadex G-75 column (2.5×64.0 cm) equilibrated and eluted with the same buffer, flowing at 18 mL/h. Fractions of 3 mL were collected and the protein content was recorded by reading absorbance at 280 nm and xylanase activity assayed as described previously. To determine xylanase molecular masses through gel filtration chromatography, the column was calibrated using blue dextran for the void volume determination and ribonuclease (15.4 kDa), chymotrypsin (25.0 kDa), ovalbumin (43.0 kDa), and bovine serum albumin (67.0 kDa) as standards. The molecular weights of xylanases were estimated from a regression curve (R^2=0.993) by plotting log of the molecular weights of the standards against the ratio between elution volumes of the standards and the void volume of the column.

Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a gradient of 8–18% (*w*/*v*) polyacrylamide according to Laemmli [17]. The resolved protein bands were visualized after staining with 0.1% Coomassie brilliant blue R-250

dissolved in methanol, acetic acid, and distilled water (4:1:5, *v*/*v*/*v*). The proteins phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, and α-lactalbumin (SDS-LMW markers—Sigma) were used to plot the standard curve log of molecular weight against relative mobility in the gel.

Purified Xylanase Characterization

Determination of Carbohydrate Concentration

Total carbohydrate was measured colorimetrically according to Dubois phenol–sulfuric acid method [18], with glucose as standard.

Temperature and pH Optima, Thermal and pH Stability

The optimum temperatures were determined by performing the reaction at temperatures ranging from 15 to 70 °C in McIlvaine buffer pH 4.5. To determine the optimum pH, the purified xylanases were assayed at 50 or 55 °C in different pH values using 0.05 M glycine–HCl buffer for pH from 1.6 to 2.5 and McIlvaine buffer from 3.0 to 7.5.

For pH stability assays, the purified enzymes were diluted (1:2 *v*/*v*) in 0.05 M glycine–HCl buffer for pH 1.6 to 2.5 and in McIlvaine buffer for pH range from 3.0 to 7.5. The samples were incubated at 4 °C for 24 h. After this period, the activities of xylanases were assayed under optimal conditions for each enzyme. To evaluate the thermal stability, the purified enzymes were incubated at different temperatures at the optimal pH determined above for different periods.

Effect of Substances

The effect of metallic ions and other compounds on the activity of the purified xylanases were evaluated at concentrations of 2 and 10 mM. The residual activities were measured in relation to the control without substances by performing the enzyme assay at the optimal conditions for each enzyme.

Substrate Specificity

Specificities of xylanases against birchwood xylan, oat spelt xylan, carboxymethyl cellulose, and avicel were assayed. Substrate solutions of 1% (*w*/*v*) were prepared in a buffer of optimum pH activity for each enzyme.

Kinetic Parameters

The enzymes were incubated with oat spelt and birchwood xylans at concentrations between 4.0 and 30 mg/mL. The Michaelis–Menten constant (K_m) and maximum reaction velocity (V_{max}) were estimated from the Lineweaver–Burk reciprocal plots, using 'GraFit' 5.0 software.

Mode of Action

The products of enzymatic hydrolysis of 1% (*w*/*v*) oat spelt xylan reaction mixtures incubated in optimal conditions for the purified xylanases were examined by thin-layer

chromatography (TLC) on silica gel G-60 precoated plates (10×15 cm), as described by Fontana et al. [19]. After 10, 30, 120, and 1,020 min of incubation at 50 °C, reaction mixtures were sampled, the enzyme activity was stopped by freezing, centrifuged, and the samples were applied on TLC plates. The mobile phase was ethyl acetate/acetic acid/formic acid/distilled water (9:3:1:4, *v*/*v*/*v*/*v*). Plates were revealed by applying of 0.2% (*w*/*v*) orcinol in sulfuric acid/methanol (1:9, *v*/*v*).

Results and Discussion

Purification of Xylanases

Conventional purification methods were effective to purify both xylanases from *P. sclerotiorum.* These enzymes were purified from the culture supernatant by chromatography on DEAE-Sephadex A-50 followed by Sephadex G-75 chromatography. After ion-exchange chromatography (Fig. 1), two protein peaks showing xylanase activity were obtained: xylanase I, not retained on DEAE-Sephadex A-50 resin, representing 63.8% of the crude filtrate activity and xylanase II, recovered by elution with a NaCl gradient, corresponding to 24.3% of the initial activity.

The pooled fractions corresponding to xylanase I and II were subsequently applied to molecular exclusion chromatography. For both samples, only one protein peak with xylanase activity was observed (Figs. 2 and 3). The fractions corresponding to these peaks were collected and the samples showed electrophoretic homogeneity (Fig. 4). After purification, xylanase I exhibited a specific activity of 249.15 U mg^{-1} protein, 2.49-fold purification, and recovery of 27.1%. Xylanase II presented a specific activity of 240.89 U mg^{-1} protein, 2.41-fold purification, and recovery of 9.8% (Table 1). Similarly to *P. sclerotiorum*,

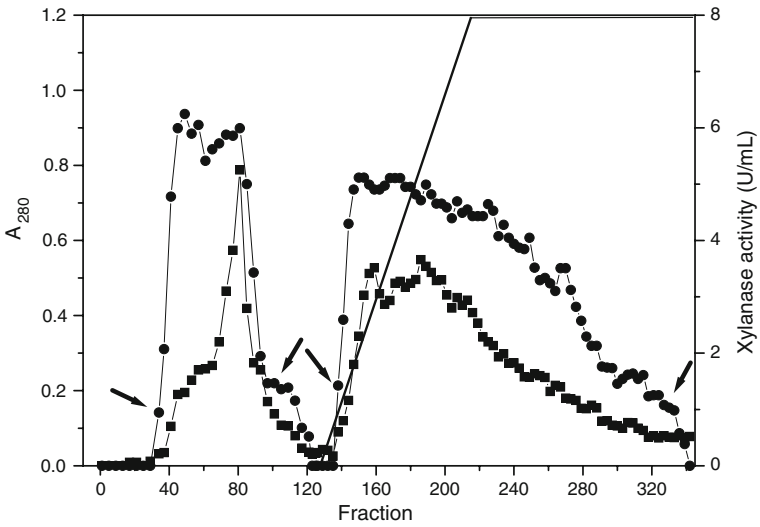

Fig. 1 DEAE-Sephadex A-50 chromatography of the xylanases from *P. sclerotiorum*. The *arrows* indicate the fractions pooled with xylanase activity. The column was equilibrated with 50 mM Tris–HCl buffer pH 9.0 and eluted with a linear salt gradient from 0.0 to 0.5 M in the same buffer. The flow rate and fraction size were 60 mL/h and 3.0 mL, respectively. (■) A_{280}, (●) xylanase activity, and (—) NaCl concentration

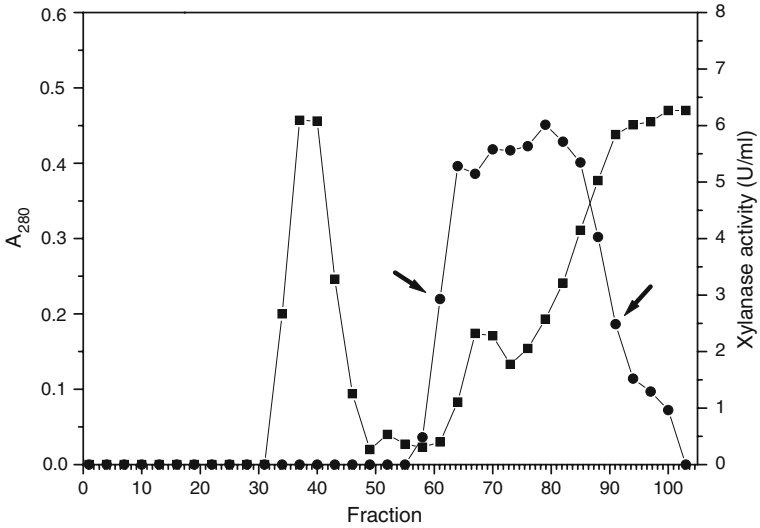

Fig. 2 Gel filtration on Sephadex G-75 of xylanase I from *P. sclerotiorum*. The *arrows* indicate the fractions pooled with xylanase activity. The column was equilibrated and eluted with 50 mM ammonium acetate buffer pH 6.8. The flow rate and fraction size were 18 mL/h and 3.0 mL, respectively. (■) A_{280}, (●) xylanase activity

many microorganisms produce a multiplicity of xylanases to achieve effective hydrolysis of xylan [4].

The molecular weights estimated by SDS-PAGE were 23.9 and 33.1 kDa for xylanase I and II, respectively. Native enzyme molecular masses of 23.8 and 30.8 kDa were estimated

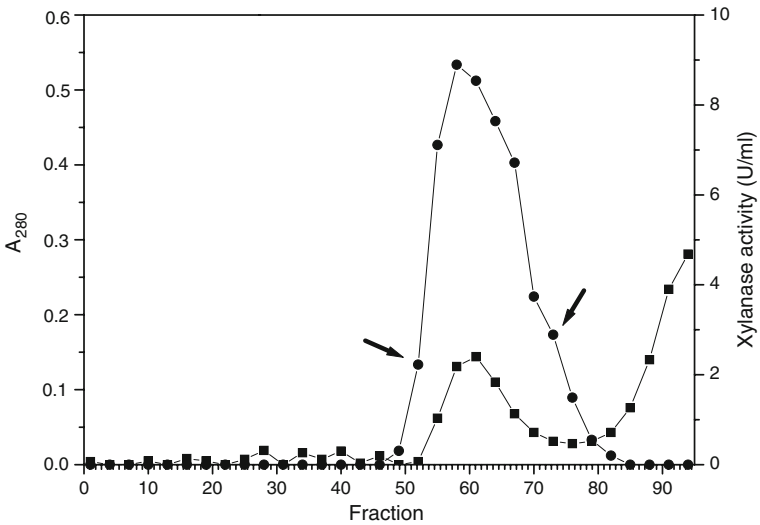

Fig. 3 Gel filtration on Sephadex G-75 of the xylanase II from *P. sclerotiorum*. The *arrows* indicate the fractions pooled with xylanase activity. The column was equilibrated and eluted with 50 mM ammonium acetate buffer pH 6.8. The flow rate and fraction size were 18 mL/h and 3.0 mL, respectively. (■) A_{280}, (●) xylanase activity

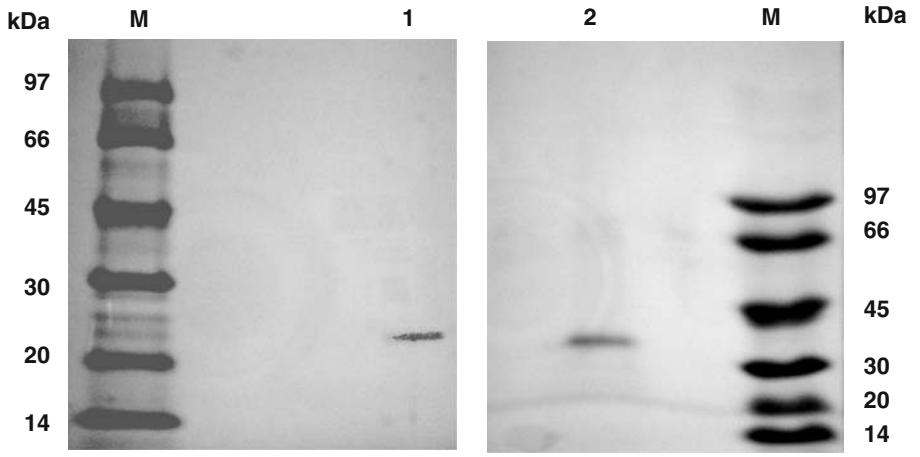

Fig. 4 SDS-PAGE (8–18%) of purified xylanase I and II from *P. sclerotiorum*. *Lane M* low molecular weight standard proteins, *lane 1* xylanase I, *lane 2* xylanase II

for xylanase I and II, respectively, by molecular exclusion chromatography, showing monomeric forms. According to Törrönen and Rouvinen [20], microbial xylanases are usually monomeric proteins and the estimated values of their molecular weights are in agreement with those found for the catalytic domain of low molecular weight xylanases, belonging to family 11. Isozymes with different molecular masses were produced by the rumen fungus *Neocallimastix frontalis*, and molecular masses of xylanases I and II were 45 and 70 kDa, respectively [21]. Nair et al. [22] purified xylanase I and II from *Aspergillus sydowii*, with molecular masses of 20.1 and 43.0 kDa, respectively. The *Aspergillus giganteus* xylanases showed molecular masses of 21 and 24 kDa [23], while 19 and 32 kDa were the molecular masses estimated for xylanases from *Aspergillus versicolor* [24, 25].

The carbohydrate contents of purified enzymes were estimated to be 14.8% for xylanase I and 65.1% for xylanase II. Glycosylation is a common feature among extracellular fungal xylanases [5]. High carbohydrate content was also verified in xylanases from *A. sydowii* (40.6% and 53.7%) [22], *Aspergillus fumigatus* (46.4% and 68.0%) [26], and *A. versicolor* (71.0%) [24]. However, these carbohydrate contents are much higher than those observed for the xylanases from *Paecilomyces themophila* (21.0%) [27], *Paecilomyces varioti* (4.5%) [28], and a minor form of *A. versicolor* xylanase (14.1%) [25]. Glycosylation provides a post-translational modification mechanism that modulates secreted enzymes. It has been

Table 1 Purification of xylanase I and II from *P. sclerotiorum.*

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Culture filtrate	8,551.7	85.40	100.14	1.00	100.0
DEAE-Sephadex A-50					
Xyl I	5,454.9	36.13	150.98	1.51	63.8
Xyl II	2,075.6	48.65	42.66	0.43	24.3
Sephadex G-75					
Xyl I	2,318.7	9.30	249.15	2.49	27.1
Xyl II	838.3	3.48	240.89	2.41	9.8

shown that glycosylation of xylanases might contribute to the stability of the protein conformation, thus increasing enzymatic activity [29].

Enzyme Properties

Effects of pH and Temperature, Thermal and pH Stabilities

The effects of temperature and pH on the activities of the purified xylanases were investigated (Fig. 5). Highest xylanase I activity was detected at 50 °C compared to the 55 °C observed for xylanase II. The optimal temperatures of the purified xylanases were similar to other xylanases of fungal origin [4, 12]. Xylanase from *Aspergillus niger* presented optimal temperature at 50 °C [30], while purified xylanases I and II from *Aspergillus caespitosus* exhibited the same optimum temperature of 50–55 °C [31]. Optimum thermal parameters for the action of xylanases from *Fusarium verticilloides* [32] and *Penicillium citrinum* [33] were also identical to those determined for xylanase I. According to the thermostability assay (Fig. 6), the xylanase II in the present investigation was more stable than xylanase I. The half-life ($T_{1/2}$) exhibited by xylanase I and II at 50 °C were 40 and 90 min, respectively. Xylanase II retained almost 80% of its activity after 90 min of incubation at 45 °C, while xylanase I was stable at 40 °C, maintaining approximately 85% of the initial activity after 90 min of incubation.

Xylanase I showed optimal activity at pH 2.5 and its activity was maintained over 80% at pH 1.6 to 3.0, while for xylanase II the optimum pH was 4.5, retaining more than 60% optimal activity in the pH range from 3.5 to 7.0 (Fig. 5b). *P. sclerotiorum* xylanase I showed activity at very low pH, one of the most acid described in literature, except for the xylanase from *Penicillium* sp. 40, with optimal activity at pH 2.0 [34]. *Laetiporus sulphureus* showed xylanase activity at an optimum pH of 3.0 [35]. However, most of the reported xylanases had an optimum pH between 5.0 and 7.0 [36] and among the acidophilic xylanases, the majority of them showed high activity under slightly acid conditions. In the pH range from 1.6 to 7.5, the purified xylanases exhibited distinct stability profiles (Fig. 7). Both enzymes exhibited high stability around its optimal pH. Xylanase I presented above 70% of its residual activity in all acidic and neutral conditions evaluated. Xylanase II remained stable in pH ranging from 2.5 to 4.5, maintaining more than 80% of its activity in these conditions and showed more than 50% of its activity in pH between 5.0 and 7.0. Microbial xylanases are usually stable over a wide pH range (3–10) and show optimum pH in the range 4.0–7.0 [2]. Studies with family 11 xylanases suggest a correlation between pH activity/stability and the number of salt bridges, with acidophilic xylanases presenting much less of these interactions than their alkalophilic homologs [37]. The optimum activity in very acidic conditions and pH stability exhibited by xylanase I make its use attractive for some industrial applications such as in feed and food industries.

Effect of Substances

In order to verify the effect of substances on xylanase activities, the purified enzymes were incubated in the presence of several metallic ions, sodium dodecyl sulfate (SDS), tetrasodium ethylenediaminetetraacetate (EDTA), dithio-treitol (DTT), phenylmethylsulphonyl fluoride (PMSF), and β-mercaptoethanol, at 2- and 10-mM concentrations (Table 2). In general, the activities of both enzymes enhanced with increased concentration of the substances used. Hg^{2+} and Cu^{2+} were strong inhibitors of both xylanases, while Zn^{2+} had a moderate inhibitory effect on xylanase I and II. Likewise, *Aspergillus fucuum*, *A. giganteus*,

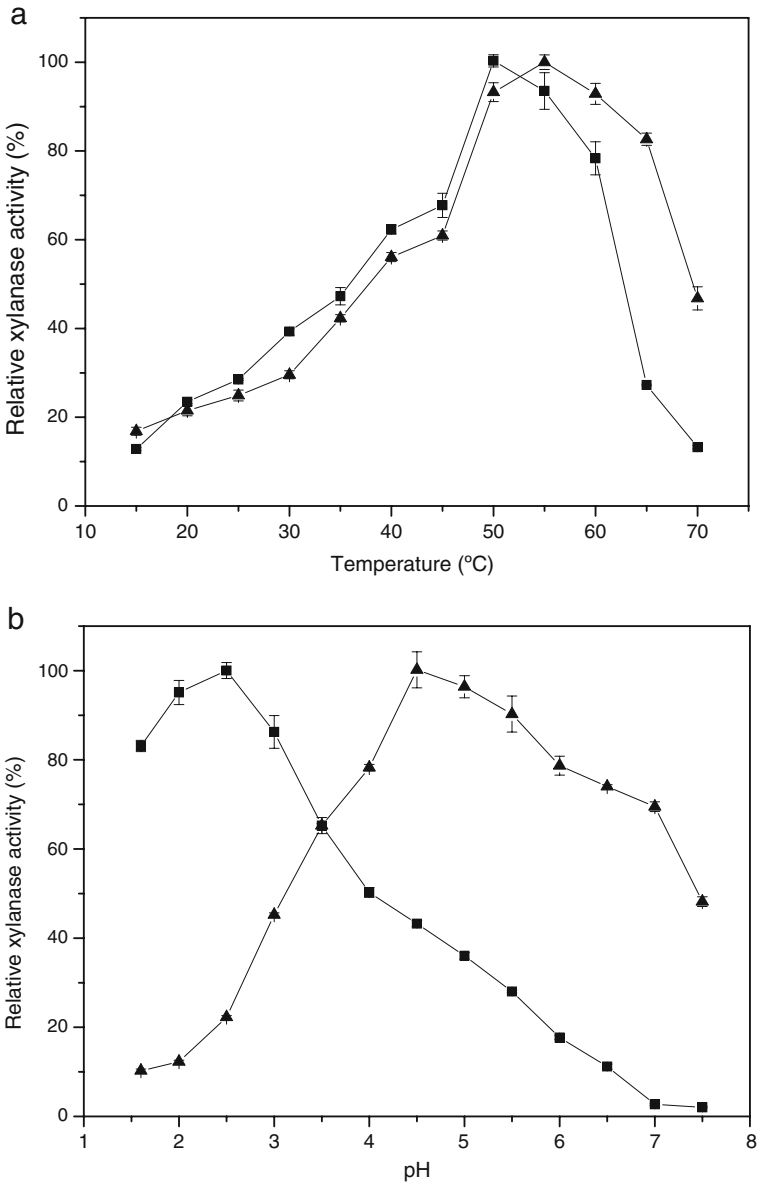

Fig. 5 Influence of temperature (**a**) and pH (**b**) on the xylanases I and II activities from *P. sclerotiorum*. Assay conditions: McIlvaine buffer pH 4.5 (**a**); 0.05 M glycine–HCl buffer from pH 1.6 to 2.5 and McIlvaine buffer from 3.0 to 7.5; 50 °C to xylanase I and at 55 °C to xylanase II (**b**). (■) Xylanase I, (▲) xylanase II

and *A. versicolor* xylanases were inhibited by Hg^{2+} and Cu^{2+} [24, 25, 38]. The inhibition by Hg^{2+} seems to be a general property of xylanases, indicating the presence of thiol groups of cysteine residues in their active sites or around them [39]. Xylanase I activity remains unaltered in the presence of Na^{+}, Mn^{2+}, and Co^{2+}, while the activity of xylanase II was not affected by Na^{+} and Mg^{2+}. Slight activation was observed for xylanase I and II in the

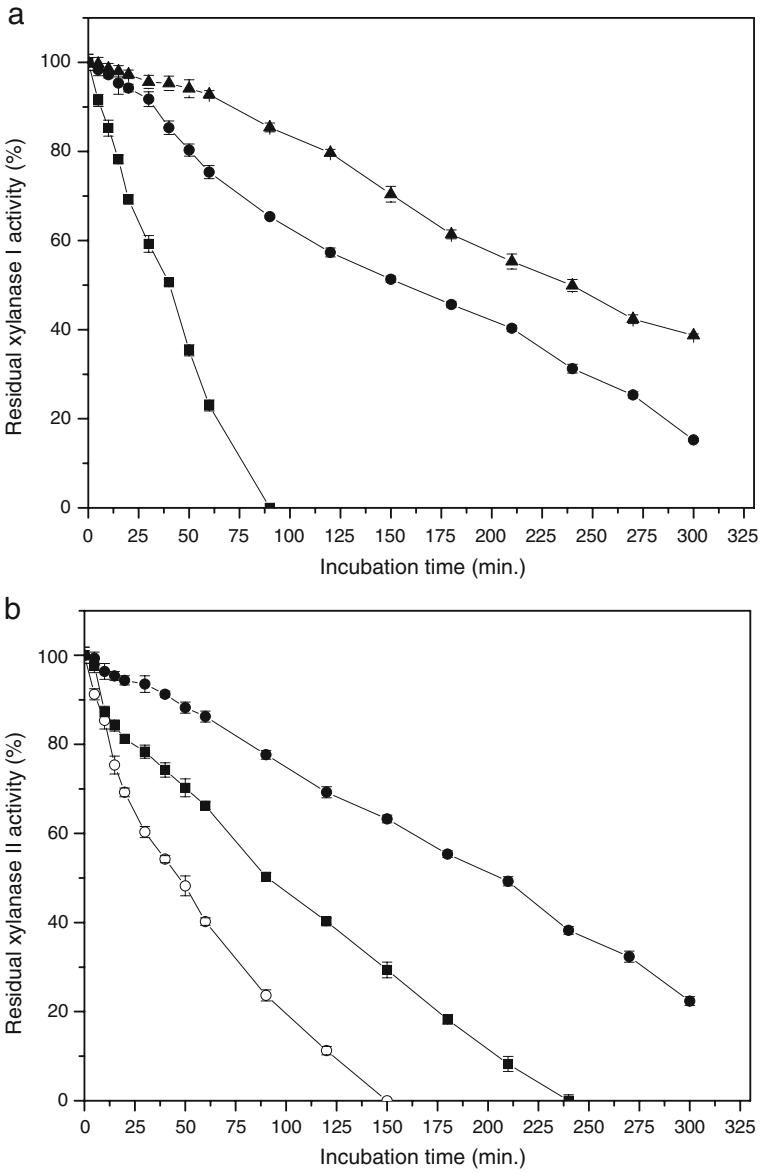

Fig. 6 Thermal stability of xylanase I (**a**) and xylanase II (**b**) activities from *P. sclerotiorum*. The purified xylanases were incubated at 40 (▲), 45 (●), 50 (■), and 55 °C (○) without substrate. Assay conditions: 0.05 M glycine–HCl buffer pH 2.5 (**a**) and McIlvaine buffer pH 4.5 (**b**)

presence of Ca^{2+} and sodium citrate. Additionally, xylanase I was activated by Ba^{2+} and NH_4^+ and xylanase II activity was remarkably stimulated when incubated with Mn^{2+}.

EDTA, a metal chelator, decreased xylanase activities, indicating that the purified enzymes require metal ions for their actions. Total loss of activity was also observed for both enzymes in the presence of SDS, suggesting that hydrophobic interactions may be important in maintaining the structures of the enzymes. The reducing agents β-

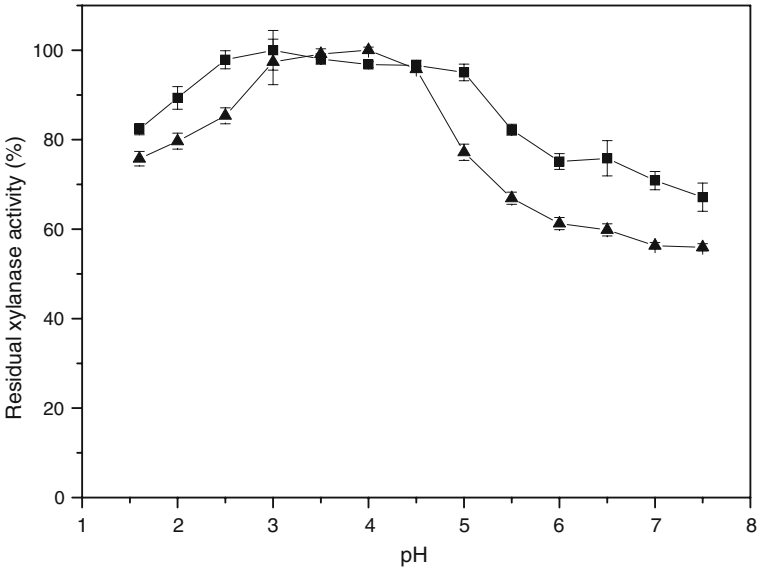

Fig. 7 pH stability of xylanase I (■) and xylanase II (▲) from *P. sclerotiorum*. The purified enzymes were pre-incubated without substrate with 0.05 M glycine–HCl buffer from pH 1.6 to 2.5 and McIlvaine buffer from pH 3.0 to 7.5, at 4 °C for 24 h

mercaptoethanol and DTT enhanced xylanase activities. The stimulation of the enzymatic activities in the presence of these thiol group protecting agents can be explained by preventing the oxidation of sulfhydryl groups. Similarly, Fialho and Carmona [23], Dutta et al. [33], and Kang et al. [40] related the involvement of cysteine residues to the maintenance of tertiary structure of the active site in *A. giganteus*, *Acrophialophora nainana*, and *P. citrinum* xylanases.

Substrate Specificity and Kinetic Studies

Specificity studies indicated that the xylanases did not hydrolyze avicel or carboxymethylcellulose, but acted only on xylans. Strong specificity toward birchwood and oat spelt xylans was also verified to xylanases from *Penicillium chrysogenum* [41] and *A. giganteus* [23]. K_m and V_{max} values were estimated using oat spelt xylan—a ramified arabinoxylan, and birchwood xylan—a xylan with few ramifications with approximately 94% of xylose [42]. The purified xylanase exhibited typical Michaelis–Menten kinetics for both substrates, allowing the corresponding kinetic constants to be calculated. Xylanase I showed K_m values of 6.5 and 2.6 mg mL^{-1} and V_{max} values of 189.70 and 241.25 μmol min^{-1} mg^{-1} of protein for birchwood and oat spelt xylans, respectively. Xylanase II exhibited K_m of 26.61 mg mL^{-1} with a V_{max} of 90.25 μmol min^{-1} mg^{-1} of protein for birchwood xylan and K_m of 23.45 mg mL^{-1} and V_{max} of 123.68 μmol min^{-1} mg^{-1} of protein for oat spelt xylan. The K_m and V_{max} values exhibited by xylanases I and II are in agreement with the values presented by other fungal xylanases which range from 0.09 to 40.9 mg mL^{-1} for K_m and from 0.106 to 6300 μM min^{-1} mg^{-1} for V_{max} [4]. The values of K_m for these two substrates indicated that both xylanases had greater affinity for the oat spelt xylan, but higher affinity for this substrate is shown by xylanase I than xylanase II. Both xylanases also showed highest V_{max} values for oat spelt xylan. Thus, both purified xylanases have

Table 2 Effect of different substances on relative activity of purified xylanases from *P. sclerotiorum*.

Substance	Xyl I activity (%)		Xyl II activity (%)	
	Concentration			
	2 mM	10 mM	2 mM	10 mM
Control	100	100	100	100
$CuCl_2$	89.8±2.1	22.5±1.7	49.7±1.4	21.9±0.5
$ZnSO_4$	82.1±0.9	65.6±1.7	85.7±1.9	63.7±1.9
$MnSO_4$	97.9±1.4	99.3±1.5	220.3±3.6	225.8±2.9
$BaCl_2$	110.2±0.8	116.3±1.3	96.0±1.2	94.6±1.1
$CaCl_2$	127.3±1.2	128.7±0.6	110.7±1.7	122.0±1.4
NH_4Cl	127.9±1.2	130.0±1.4	80.7±1.8	75.2±1.6
NaCl	99.7±1.4	99.6±0.8	102.5±1.3	101.3±1.7
SDS	ND	ND	13.9±0.3	ND
PMSF	114.6±0.7	118.6±1.5	70.4±1.5	64.2±0.9
$MgSO_4$	95.5±2.1	93.2±1.5	98.6±1.3	100.6±1.4
Sodium citrate	108.5±1.3	124.5±2.3	111.6±2.1	118.0±1.1
DTT	141.7±3.2	193.8±3.4	148.4±2.4	160.2±3.8
$CoCl_2$	102.7±1.2	103.3±1.5	42.7±1.2	ND
$HgCl_2$	50.7±0.9	ND	63.9±0.9	ND
$Pb(CH_3COO)_2$	99.6±1.1	76.3±2.1	99.2±1.2	112.8±0.9
EDTA	94.8±0.9	63.0±1.0	80.7±1.1	27.9±0.5
β-Mercaptoethanol	143.1±3.1	180.3±2.8	109.6±2.1	128.7±2.3

higher catalytic efficiencies for hydrolyzing oat spelt xylan. Similarly, xylanase II from *A. giganteus* [23] exhibited higher affinity for oat spelt xylan and xylanase from *Fusarium oxysporum* [43] and xylanase III from *A. nainana* [44] showed highest value of V_{max} for oat spelt xylan.

Mode of Action

The hydrolysis products of the purified xylanases were studied by ascending TLC analysis in reaction mixture incubated for a period up to 17 h (Fig. 8). The mobility of hydrolysis products in relation to xylose was similar to that described by Fontana et al. [19], using the same solvent system, revealing xylotriose, xylotetraose, and larger xylooligosaccharides formation. Xylanase I released xylobiose and other larger xylooligosaccharides, while xylanase II apparently only liberated xylooligosaccharides decreasing in polymerization degree up to xylotriose. Xylotriose is the smallest oligomer produced by most of the known xylanases [2]. Hence, these enzymes may be classified as endoxylanases.

Potential Biotechnological Applications

The purification and characterization of xylanolytic system from *P. sclerotiorum* indicate that xylanase I is a novel enzyme with interesting biochemical properties that make it a potential candidate for industrial and commercial application in feed and food industries. Of

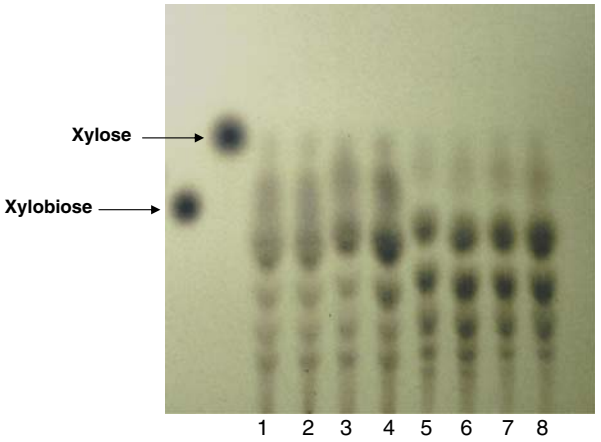

Fig. 8 Thin-layer chromatography of the hydrolysis products of oat spelt xylan from xylanases I and II. *Lanes 1*, *2*, *3*, and *4* show the hydrolysis products of xylanase I after 10, 30, 120, and 1,020 min of incubation, respectively; *lanes 5*, *6*, *7*, and *8* show the hydrolysis products of xylanase II after 10, 30, 120, and 1,020 min of incubation, in that order

particular interest is the fact that it shows an unusual optimum pH at 2.5 and remarkable stability at acidic conditions. One of the specific applications is in animal feed industry. Several studies demonstrated that the incorporation of xylanase into diet of animals results in the reduction of intestinal viscosity, thereby improving weight gain and increasing feed conversion [45]. Xylanases with low pH optimum and high pH stability in acid pH would be most suitable for animal feed due to extreme pH prevalence in the digestive tract. Through the application of acid-stable enzymes, the nutrient availability in ruminant diets can be increased [46]. Most of the commercially available enzymatic products that have been tested as food additives for ruminants were not designed specifically for this purpose; enzymatic preparations containing cellulases and xylanases destined for use in the food, pulp, paper, textile, fuel, and other chemical industries have been used [47]. Despite xylanase I exhibiting lower levels of activity at animal's regular temperature, its combination with the substrate can significantly increase its performance. In addition, an increase of activity and thermal stability in this temperature can be achieved with site-directed mutagenesis. Thermal stability is the most commonly addressed stability parameter, whereas the structural basis of extreme pH stability is less well understood. Extreme pH activity and stability is also more difficult to be improved [48]. Specific applications for *P. sclerotiorum* xylanase I also include its use in clarification and maceration of juices and wines [49]. The extremely acid pH of must and wines limited the use of the enzymes in this process [50]. For a practical purpose, it may be advantageous and efficient to utilize the acid xylanase obtained from this organism under harsh conditions in feed and food industries.

In addition, the other attractive biochemical characteristic which can be further explored is the xylobiose release through xylanase I action on xylan. For food applications, xylobiose and xylooligosaccharides have been used as a food ingredient to modulate the intestinal function since they could be selectively used by the beneficial gastrointestinal microbiota and suppress the growth of pathogenic bacteria [51]. The production of xylobiose is a time-consuming and expensive process [52]. The synthesis of xylooligosaccharides using hydrolytic enzymes such as xylanases is receiving great attention [53–55] and emerges as a great alternative to obtain these products.

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