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Purification and characterization of xylanases from *Trichoderma inhamatum*

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ABSTRACT

Background: Two xylanases, Xyl I and Xyl II, were purified from the crude extracellular extract of a *Trichoderma inhamatum* strain cultivated in liquid medium with oat spelts xylan.
Results: The molecular masses of the purified enzymes estimated by SDS-PAGE and gel filtration were, respectively, 19 and 14 kDa for Xyl I and 21 and 14.6 kDa for Xyl II. The enzymes are glycoproteins with optimum activity at 50°C in pH 5.0–5.5 for Xyl I and 5.5 for Xyl II. The xylanases were very stable at 40°C and in the pH ranges from 4.5–6.5 for Xyl I and 4.0–8.0 for Xyl II. The ion Hg^{2+} and the detergent SDS strongly reduced the activity while 1,4-dithiothreitol stimulated both enzymes. The xylanases showed specificity for xylan, K_m and V_{max} of 14.5, 1.6 mg·mL^{-1} and 2680.2 and 462.2 U·mg of protein^{-1} (Xyl I) and 10.7, 4.0 mg·mL^{-1} and 4553.7 and 1972.7 U·mg of protein^{-1} (Xyl II) on oat spelts and birchwood xylan, respectively. The hydrolysis of oat spelts xylan released xylobiose, xylotriose, xylotetrose and larger xylooligosaccharides.
Conclusions: The enzymes present potential for application in industrial processes that require activity in acid conditions, wide-ranging pH stability, such as for animal feed, or juice and wine industries.

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1. Introduction

Xylans are a diverse and complex group of polysaccharides with the common feature of a backbone of β-(1 → 4)-linked xylose residues [1], in which side-chains are attached at the C2 and C3 positions of D-xylosyl. These substituents can normally be acetyl, 4-*O*-methyl-D-glucuronosyl or L-arabinosyl groups [2,3]. Xylan is the predominant hemicellulose found in plant cell walls strongly associated to cellulose microfibrils and the strength of this interaction is inversely related to the degree of substitution of the main chain by side-groups [4].

The conversion of xylan into useful products represents a significant part of the effort to achieve economical viability of the lignocellulose biomass processing and to develop different ways to produce chemicals and renewable energy as well. Owing to its complex structure, the complete degradation of xylan requires the joint of several hydrolytic enzymes acting in synergy that is known as a xylanolytic system. Endo-β-1,4-xylanases (β-1,4-D-xylan xylanohydrolases, EC 3.2.1.8) are the most important xylanolytic enzymes, cleaving internal glycoside bonds in xylan backbone, reducing the degree of polymerization of the polymer [5,6]. The cleavage carried out by these enzymes is not aleatory, *i.e.* side chain decorations in xylan are recognized by xylanases, and the degree of substitution in the polymer influences the product of hydrolysis. This difference in substrate specificity among different xylanases has important implications in the deconstruction of xylan [3].

Interest in xylanolytic enzymes has increased in last decades due to their industrial applications in the food, feed, and pharmaceutical industries and for sustainable production of fuels and chemicals. Besides, they can be applied in some processes in which cellulolytic activity must be absent, to preserve the vegetal fibers, in the pulp and paper industries, and in the processing of flax, hemp and jute in the textile industries [7,8,9].

Fungi are commonly used as source of xylanases and their xylanolytic systems have been widely studied [6,10]. *Trichoderma* spp. xylanases are among the most known enzymes, therefore, this fungal genus is suited for further examination of function and application of these enzymes [11]. *Trichoderma reesei* Rut C-30 is the most well-known *Trichoderma* strain producing several xylanases and cellulases with different biochemical properties and specificities for substrates, as predicted by genome sequence [12], and also many enzyme preparations obtained from the large-scale cultivation of this fungus have been commercialized. Recently, rational and efficient systems for the production of cocktails containing different balances between xylanolytic and cellulolytic enzymes have been investigated for the different application of these enzymes [13]. Xylanases from other *Trichoderma* species have also been studied as those from *Trichoderma harzianum*, *Trichoderma lignorum*, *Trichoderma longibrachiatum*, *Trichoderma koningii*, *Trichoderma pseudokoningii* and *Trichoderma viride*

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[14,15]. Other xylanases, including that from a psychrotrophic *Trichoderma* strain have been purified and characterized [16], and also xylanase encoding genes from other *Trichoderma* species have been isolated, cloned and expressed in *Escherichia coli* [17], *Saccharomyces cerevisiae* [18] and *Pichia pastoris* [19].

When a new efficient xylanase-producing microorganism is isolated, it is essential to purify and characterize the enzymes to know the action towards substrates of each component of a xylanolytic complex, its regulation and biochemical properties in order to develop more competitive processes. A *Trichoderma inhamatum* strain, isolated from soil in São Paulo state (Brazil), produces high levels of xylanase in the absence of cellulases [20], an important condition for some industrial applications, as stated above. The influence of some parameters affecting xylanase production by this fungal strain has already been investigated [21]. The present study aimed to purify and characterize the main xylanases produced by this fungus under previously optimized culture conditions.

2. Materials and methods

2.1. Fungal strain: maintenance and culture conditions

T. inhamatum was deposited in the Environmental Studies Center Collection, CEA/UNESP, Brazil. The fungal strain was maintained on Vogel solid medium slants [22] with 1.5% (w/v) wheat bran, at 4°C and cultured periodically. The cultures were inoculated in the same medium with 1.5% (w/v) glucose and incubated for conidia production during 7 days at 28°C. Conidia were harvested, suspended in sterile distilled water and the concentration of the suspension was adjusted to 10^7 conidia milliliter^{-1}. One milliliter of this suspension was inoculated into Vogel liquid medium pH 6.0 with 1% (w/v) oat spelts xylan as sole carbon source for enzyme production. Cultures were maintained in orbital shaker (120 rpm) at 25°C for 60 h [21]. After cultivation, mycelium was removed by vacuum filtration and the crude culture filtrate was used as source of enzymes.

2.2. Enzyme assay

Xylanase activity was determined by incubating enzyme samples with 1% (w/v) birchwood xylan (Sigma, USA; xylose residues ≥90%) in 0.05 M sodium acetate buffer pH 5.5 at 50°C. At suitable intervals, the reaction was interrupted with 3,5-dinitrosalicylic acid (DNS) reagent and the released reducing sugars were measured [23], using xylose as standard. One unit of activity (U) was defined as the amount of enzyme capable to release 1 μmol of reducing groups per min. Specific activity was expressed by the relation between enzyme activity and protein content.

2.3. Determination of protein and carbohydrate

Protein was determined by the Lowry method [24], with bovine serum albumin as standard. During the chromatographic steps, proteins were detected by reading absorbance at 280 nm. Total carbohydrate was determined by the phenol-sulphuric acid method [25], with glucose as standard.

2.4. Purification of xylanases

The crude culture filtrate (200 mL) was dialyzed against 0.05 M Tris–HCl buffer pH 7.0 for 8 h, with buffer changes each 2 h, in order to exclude small molecules and obtain a buffered solution in this pH. This sample was submitted to ion exchange chromatography on a DEAE Sephadex A-50 column (1.1 × 14.5 cm), previously equilibrated with the same dialysis buffer; at 50 mL/h flow rate. Adsorbed proteins were then eluted with a 0.0–0.5 M NaCl linear gradient in the same buffer. Proteins were detected by reading absorbance at 280 nm and xylanase activity was assayed in the collected 3 mL fractions. Fractions with high xylanase activity were pooled and submitted to electrophoresis (SDS-PAGE). The sample corresponding to the not retained fractions was further dialyzed against 0.05 M ammonium acetate buffer pH 6.8 for 8 h, with buffer changes each 2 h, and then lyophilized. The sample was re-dissolved in a small volume of this buffer and applied to size exclusion chromatography on a Sephadex G-75 (1.8 × 60.5 cm) column equilibrated and eluted in the same buffer, at 19 mL/h flow rate. Proteins were detected by reading absorbance at 280 nm and xylanase activity was assayed in the collected 3 mL fractions. Fractions with high xylanase activity were pooled and submitted to electrophoresis (SDS-PAGE). All purification steps were carried out at 4°C.

2.5. Biochemical characterization of the purified xylanases

2.5.1. Electrophoresis

SDS-PAGE was performed in 8–18% (w/v) gradient gels [26] using a Mini-PROTEAN® Electrophoresis System (Bio-Rad, USA). Resolved protein bands were visualized after staining with 0.1% (w/v) Coomassie brilliant blue R-250 dissolved in methanol, acetic acid, and distilled water (4:1:5; v/v/v). The standard proteins (Sigma, USA) phosphorylase B (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20 kDa), and α-lactalbumin (14.2 kDa) were used to plot the standard curve log of molecular mass against relative mobility of the proteins.

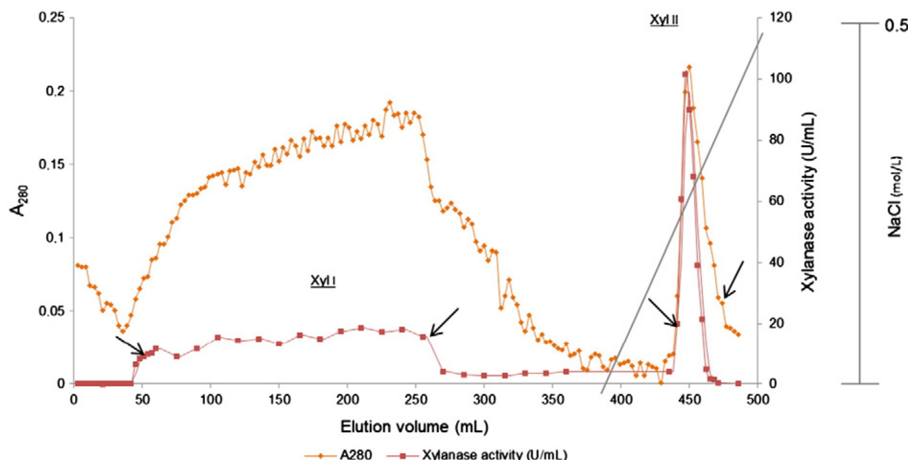

Fig. 1. DEAE Sephadex A-50 chromatography of the xylanase from *T. inhamatum*. Conditions: 0.05 M Tris–HCl buffer pH 7.0; elution with a linear salt gradient in the same buffer; flow rate 50 mL/h, fraction 3.0 mL. (-) NaCl concentration (mol/L). Arrows indicate the pooled fractions (Fractions 18–89 for Xyl I and 145–155 for Xyl II).

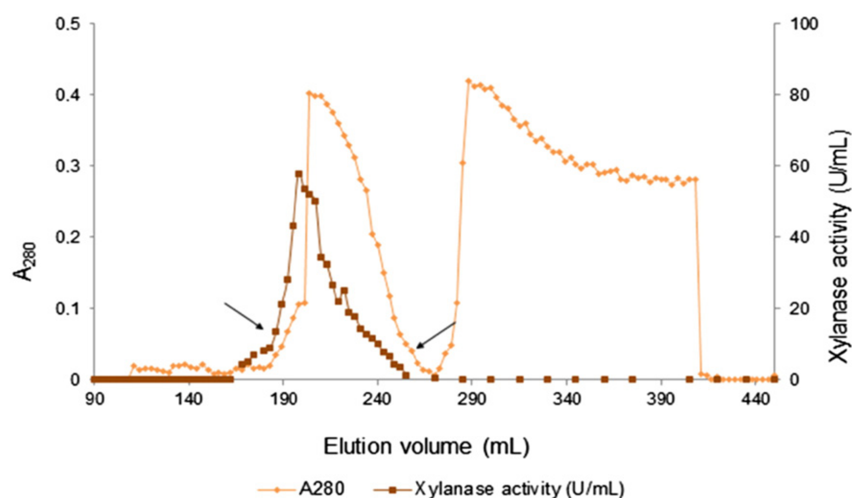

Fig. 2. Gel filtration on Sephadex G-75 of the Xyl I from *T. inhamatum*. Conditions: 0.05 M ammonium acetate buffer pH 6.8; flow rate 19 mL/h, fraction 3.0 mL. Arrows indicate the pooled fractions (Fractions 60–83).

2.5.2. Determination of molecular mass under non-denaturing conditions

Molecular masses of the proteins were estimated by gel filtration from a regression curve by plotting log of the molecular masses of the standards against the ratio between their elution volumes and the void volume (V_0), estimated using blue dextran. Standard proteins (Sigma, USA) were ribonuclease (15.4 kDa), chymotrypsin (25.0 kDa), ovalbumin (43.0 kDa) and bovine serum albumin (67.0 kDa).

2.5.3. Temperature and pH optima, stability in different temperature and pH

The best pH for activity of the purified xylanases was determined by assaying enzymatic reactions in McIlvaine buffer adjusted to various pH between 3.0 and 8.0, with 0.5 unit intervals at 50°C. For the optimal temperature, enzymatic reactions were carried out with the purified enzymes in 0.05 M sodium acetate buffer pH 5.5 at temperatures from 20 to 60°C, with 5°C intervals.

Thermal stability was determined by verifying residual activity after incubating samples of the purified enzymes without substrate at 40, 50 and 60°C during different periods. The pH stability was determined by verifying the remaining activity after incubating the purified enzymes for 24 h at room temperature. Diluted (1:2; v/v) enzyme samples were incubated with McIlvaine buffer in the pH range from 2.5 to 8.0, with 0.5 unit intervals.

2.5.4. Effect of substances on enzyme activity

The effect of substances was verified by assaying xylanase activity with a variety of compounds dissolved in 0.05 M sodium acetate buffer pH 5.5. The following substances were evaluated at 2 and 10 mM: lead acetate, ammonium chloride, barium chloride, calcium chloride, cobalt chloride, copper chloride, mercury chloride, iron sulfate, magnesium sulfate, manganese sulfate, zinc sulfate, tetrasodium ethylenediaminetetraacetate (EDTA), 1,4-dithiothreitol (DTT), sodium dodecyl sulfate (SDS) and phenylmethylsulfonyl fluoride (PMSF). All assays were carried out in triplicate.

2.5.5. Substrate specificity

The specificity of the xylanases was verified by assaying the activity against different substrates. Xylanase activity was measured on birchwood and oat spelts xylans. Endoglucanase (CMCase) and exoglucanase (Avicelase) activities were assayed in a reaction mixture with carboxymethylcellulose (CMC) and microcrystalline cellulose (Avicel), respectively. Substrates at 1% (w/v) were prepared in 0.05 M sodium acetate buffer pH 5.5 and appropriately diluted enzyme solution. Reducing sugars were quantified with the DNS acid reagent and the absorbance was measured at 540 nm. One unit of activity was defined as the amount of enzyme required to release 1 μmol of product equivalent per min in the assay conditions at 50°C.

2.5.6. Kinetic parameters

The enzymes were incubated with xylan from birchwood and from oat spelts (Sigma, USA; arabinose residues ≤10%, glucose residues ≤15%, xylose residues ≥70%) at concentrations varying from 4.0 to 30.0 mg·mL^{-1}. The Michaelis–Menten constant (K_m) and maximal velocity (V_{max}) were estimated from Lineweaver–Burk reciprocal plots [27]. Three experiments were carried out for each substrate concentration in triplicate and the straight line plotted was calculated by linear regression (Microsoft Office Excel, version 12.0) using mean values obtained from each experimental point.

2.5.7. Determination of hydrolysis products

The products of the enzymatic hydrolysis of oat spelts xylan were analyzed by thin-layer chromatography (TLC) on silica-gel G-60 plates (10 × 15 cm), using xylose and xylobiose solutions (1 mg/mL) as standards [28]. The mobile phase was ethyl acetate/acetic acid/formic

Table 1
Purification of the xylanases from *T. inhamatum*.

Step		Activity[a] (U·mL^{-1})	Volume (mL)	Total activity (U)	Total protein (mg)	Specific activity (U·mg prot^{-1})	Yield (%)	Purification (Fold)
Crude dialyzed filtrate		121.32	215	26,083.8	40.0	652.1	100	1.0
DEAE Sephadex A-50								
	Xyl I	74.32	220	16,350.4	13.0	1257.7	62.7	1.9
	Xyl II	60.82	16	973.1	0.8	1216.4	3.7	1.9
Sephadex G-75								
	Xyl I	46.19	67.5	3,117.8	0.9	3464.2	12.0	5.3

[a] The activity was assayed with 1% (w/v) birchwood xylan in 0.05 M sodium acetate buffer pH 5.5 at 50°C.

acid/distilled water (9:3:1:4; v/v/v/v). Plates were revealed by applying a 0.2% (w/v) orcinol in sulfuric acid/methanol (1:9; v/v) solution. Substrate hydrolysis by each enzyme was carried out in 0.05 M sodium acetate buffer pH 5.5 at 50°C for 10 min, 30 min, 2 h and 17 h.

3. Results and discussion

3.1. Purification of T. inhamatum xylanases

Two xylanases were purified from the crude filtrate after growth of *T. inhamatum* in liquid cultures with xylan, under optimized culture conditions. Xyl II was purified to electrophoretic homogeneity by a single step of ion exchange chromatography, while Xyl I purification required a subsequent molecular exclusion chromatography with Sephadex G-75. The first step (Fig. 1) revealed two protein peaks with xylanolytic activity: Xyl I, corresponding to the not retained fraction, presented 62.7% of the activity, and Xyl II, the retained fraction was eluted with a NaCl gradient and presented 3.7% of the activity.

The sample corresponding to the first xylanase peak, considered the main xylanase produced by *T. inhamatum*, was subsequently subjected to molecular-exclusion chromatography (Fig. 2), giving rise to two protein peaks, one of them with xylanase activity, representing 12.0% of the initial activity. Xyl I and Xyl II from *T. inhamatum* were obtained with final specific activities of 3464.2 and 1216.4 U·mg $prot^{-1}$ and 5.3 and 1.9-fold purification, respectively (Table 1).

The occurrence of multiple enzyme forms is a common phenomenon and can be considered a specialized function of microorganisms to achieve a more effective hydrolysis of heterogeneous substrates in the nature [2]. Many xylanase forms are produced by *T. reesei* Rut C-30, for example. The two major xylanases produced by that fungus are Xyn1 and Xyn2, with the latter representing more than 50% of the total xylanolytic activity and both of them are responsible for more than 90% of the specific xylanase activity produced when the fungi was grown on cellulose or xylan [29]. Besides, the presence of several minor xylanases has also been demonstrated [30].

3.2. Physico-chemical properties of T. inhamatum xylanases

SDS-PAGE revealed a single band in each sample after the purification steps. The molecular masses estimated by this method were 19 kDa for Xyl I and 21 kDa for Xyl II (Fig. 3). The molecular masses estimated under non-denaturing conditions with Sephadex G-75 were 14.0 and 14.6 kDa for Xyl I and Xyl II, respectively. These values of molecular mass are similar to those observed for many *Trichoderma* xylanases [14]. The purified xylanases were highly glycosylated, presenting 79% and 62% of carbohydrates for Xyl I and Xyl II, respectively, which justifies some distortion observed in SDS-PAGE.

The activity profiles of Xyl I and Xyl II (Fig. 4a) showed that both enzymes present activity in slightly acid region with optimal activity in pH 5.0–5.5. Both enzymes showed more than 50% of the maximum activity in the pH range from 4.5 to 6.5, and the activity decreased sharply from this range. Optimal activity was observed at 50°C for Xyl I and at 45°C–55°C for Xyl II (Fig. 4b), the former presented more than 50% of the activity between 40°C and 55°C, and the latter between 35°C–60°C. Comparatively, the optima pH and temperature observed for the *T. inhamatum* xylanases are in accordance with those from many other xylanases from mesophilic *Trichoderma* strains that are commonly observed in the pH range from 3.5 to 6.0 and in temperatures from 45 to 60°C [14].

Both enzymes were stable at 40°C (Fig. 5), *i.e.* Xyl I retained 71% and Xyl II retained 77.5% of the activity after 4.5 h of incubation. Xyl I exhibited half-life of 4 min at 50°C and of 40 s at 60°C, while the half-life of Xyl II was 18 min at 50°C and 46 s at 60°C. The purified *Trichoderma* sp. K9301 xylanase was very stable at 50°C but also rapidly lost activity when incubated at 60°C [15]. The purified xylanases exhibited distinct stabilities in pH from 3.0 to 8.0 (Fig. 6). After 24 h incubation, residual activity of 90% or more was detected in pH from 4.5 to 6.5 for Xyl I, and from 4.0 to 8.0 for Xyl II.

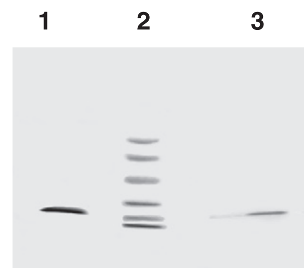

Fig. 3. SDS-PAGE (8–18%) of purified Xyl I and Xyl II from *T. inhamatum*. Lane 1: Xyl I; Lane 2: Standard proteins: phosphorylase B (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa) and α-lactalbumin (14.4 kDa); Lane 3: Xyl II.

The effect of organic compounds, metallic ions and a chelating agent on the activity of the purified xylanases from *T. inhamatum* is presented in Table 2. The ion Hg^{2+} was a strong inhibitor of the xylanases even at 2 mM, while Cu^{2+} also inhibited both enzymes, but this effect was more pronounced only at 10 mM. The inhibition by Hg^{2+} seems to be a general property of xylanases, indicating the presence of cysteine thiol groups near or in the active site of the enzyme [31]. The denaturation caused by the detergent SDS resulted in loss of the activity and, in low concentration (2 mM), the effect was more pronounced on Xyl I. The

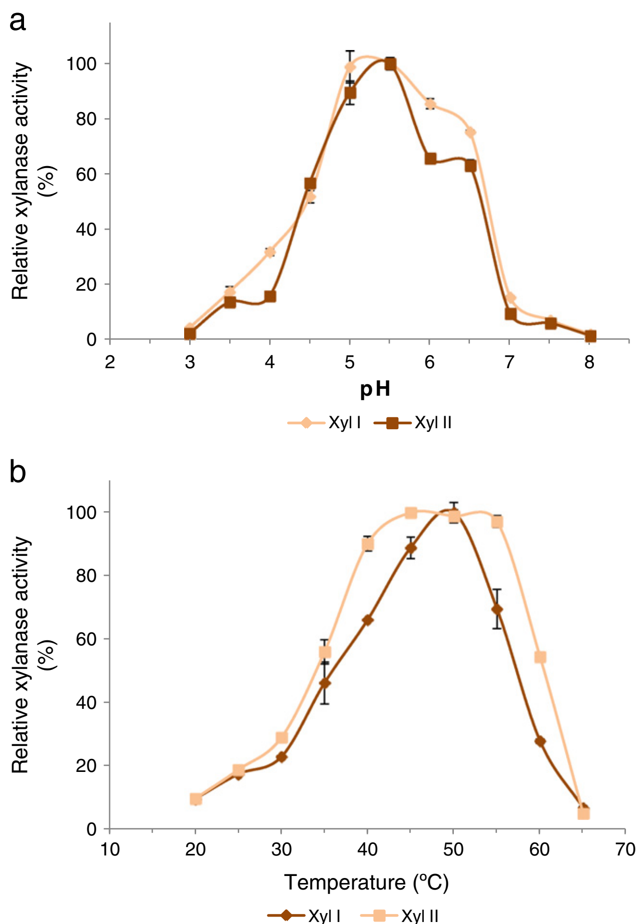

Fig. 4. Influence of (a) pH and (b) temperature on the activity of Xyl I and Xyl II from *T. inhamatum*. Assay conditions: (a) 1% (w/v) birchwood xylan in McIlvaine buffer pH 3.0–8.0, 50°C. (b) 1% (w/v) birchwood xylan in 0.05 M sodium acetate buffer pH 5.5.

ions Ca^{2+}, Co^{2+} and Mg^{2+} presented a different effect on Xyl I and Xyl II, *i.e.* activated Xyl I but inhibited Xyl II; while Mn^{2+} activated only Xyl II, with no effect on Xyl I. Besides, Xyl I was not affected by NH_4^+, Ba^{2+}, Fe^{2+}, Zn^{2+} or Mn^{2+}, while Xyl II was weakly inhibited by NH_4^+, Ba^{2+} and Fe^{2+} and moderately inhibited by Zn^{2+} only at 10 mM·Pb^{2+} inhibited the enzymes with more pronounced effect on the Xyl II at 10 mM. EDTA at both concentration and PMSF at 10 mM slightly decreased the activity of both enzymes. The effect of EDTA on both enzymes suggests that they may require divalent ion for catalysis. DTT increased the activity of both enzymes, which can be explained by the prevention of the oxidation of sulfhydryl groups in the presence of this agent or by the reduction of disulfide bridges, restoring their native structure in some specific region or even of the catalytic site.

3.3. Substrate specificity and kinetic parameters

The purified enzymes hydrolyzed exclusively xylans with no activity on Avicel or CMC. The activities of Xyl I and Xyl II on oat spelts xylan, a branched arabinoxylan were, respectively, 8% and 16% higher than those on birchwood xylan, which is as less branched xylan with 94% xylose residues [32], indicating the preference of the enzymes for branched and heterogeneous xylan. Both enzymes exhibited Michaelis–Menten kinetics and the corresponding apparent constant values were calculated (Table 3). The K_m values indicated that both enzymes had higher affinity for birchwood than for oat spelts xylan.

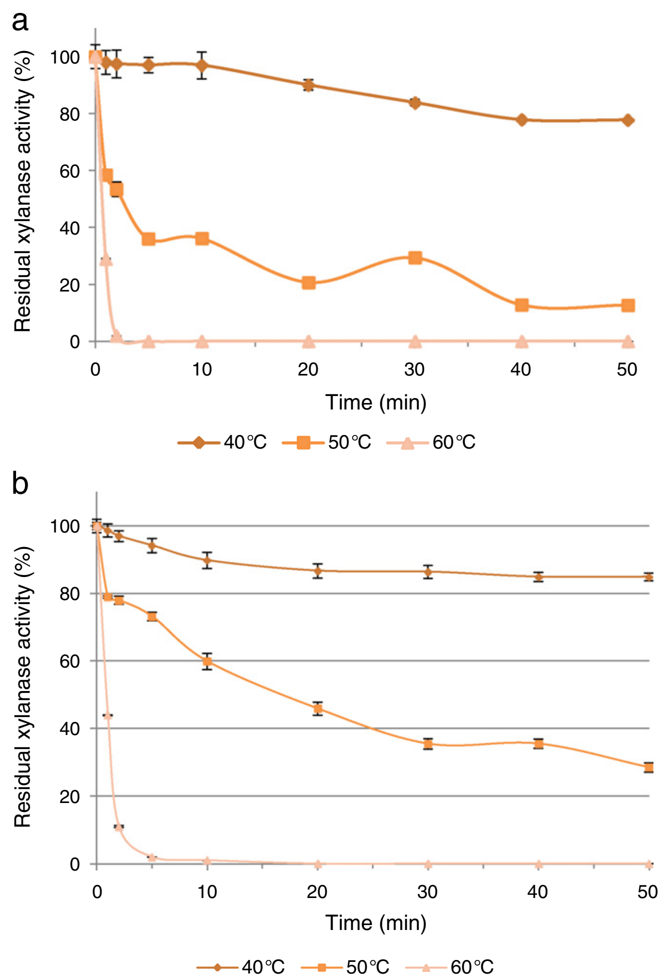

Fig. 5. Thermal stability of Xyl I (a) and Xyl II (b) from *T. inhamatum*. Conditions: the purified enzymes were incubated without substrate at 40, 50 and 60°C. The activity was assayed with 1% (w/v) birchwood xylan in 0.05 M sodium acetate buffer pH 5.5 at 50°C.

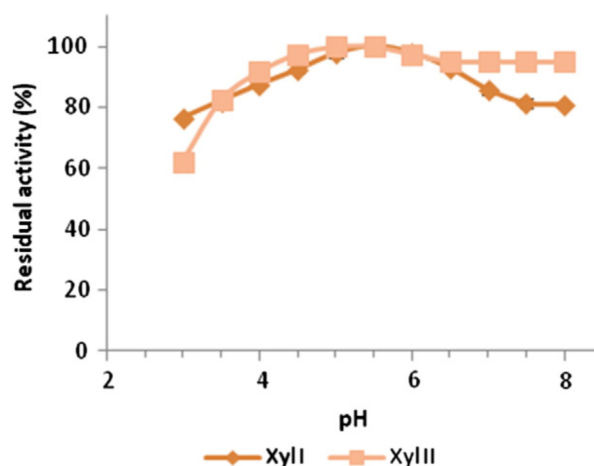

Fig. 6. Stability on different pH of Xyl I and Xyl II from *T. inhamatum*. Conditions: the purified enzymes were incubated without substrate in McIlvaine buffer pH 3.0–8.0, for 24 h at room temperature, and the activity was assayed with 1% (w/v) birchwood xylan in 0.05 M sodium acetate buffer pH 5.5 at 50°C.

With birchwood xylan as substrate, Xyl I presented the lower K_m and therefore higher affinity, while with oat spelts xylan, Xyl II had more affinity. For both substrates, the higher value of k_{cat} was that of Xyl II; for both enzymes, higher values of k_{cat} and V_{max} were observed with oat spelts xylan. The ratio k_{cat}/K_m for birchwood xylan was 19.9 and 21.7 $s^{-1}·mM^{-1}$ for Xyl I and Xyl II respectively, demonstrating similar efficiencies to hydrolyze this substrate. However, with oat spelts xylan, this ratio was about 2.5-fold greater for Xyl II than for Xyl I, indicating that the former enzyme is much more efficient in degrading this xylan. Two xylanases from *Aspergillus giganteus* [33] have similar catalytic efficiencies on these substrates, while the *Penicillum capsulatum* xylanase is 2.7-fold more efficient in hydrolyze oat spelts than birchwood xylan [34].

The mode of action was investigated by identifying the main products of oat spelts xylan hydrolysis by the purified xylanases. Since both enzymes gave products with the same R_f values over various incubation intervals, only the TLC profile obtained with Xyl I is shown in Fig. 7. Both enzymes released xylobiose and larger xylooligosaccharides and, thus, they may be classified as endoxylanases. Interestingly, not even after 17 h, release of xylose was verified, in contrast to the xylose release observed after long-term incubation [16]. According to the

Table 2
Effect of different substances on Xyl I and Xyl II from *T. inhamatum*.

Substance	Xyl I		Xyl II	
	2 mM	10 mM	2 mM	10 mM
Control	100.0%	100.0%	100.0%	100.0%
NH_4Cl	95.2 ± 5.9	100.8 ± 0.6	94.4 ± 7.0	76.7 ± 1.3
$BaCl_2$	100.1 ± 3.5	103.6 ± 0.2	85.0 ± 3.0	78.6 ± 0.0
$HgCl_2$	14.4 ± 0.0	4.0 ± 0.1	15.6 ± 0.7	5.9 ± 2.3
$CaCl_2$	109.4 ± 0.5	114.2 ± 7.8	88.3 ± 1.0	74.8 ± 4.0
$CuCl_2$	91.9 ± 0.7	20.9 ± 0.2	78.1 ± 3.4	17.0 ± 4.0
$CoCl_2$	118.1 ± 1.9	127.2 ± 2.9	77.2 ± 0.7	68.4 ± 1.0
$MgSO_4$	104.7 ± 5.6	139.1 ± 3.8	98.2 ± 5.7	59.6 ± 4.0
$FeSO_4$	95.3 ± 2.9	93.2 ± 6.5	94.4 ± 0.3	76.2 ± 8.0
$ZnSO_4$	98.0 ± 1.8	93.7 ± 1.0	90.4 ± 0.7	52.1 ± 2.7
$MnSO_4$	101.9 ± 2.4	102.5 ± 5.4	97.8 ± 1.8	133.5 ± 6.7
SDS	12.9 ± 0.4	ND	39.1 ± 3.7	ND
EDTA	91.6 ± 5.4	87.5 ± 2.9	90.4 ± 2.0	87.6 ± 3.4
$Pb(H_3C_2O_2)_2$	79.9 ± 1.3	73.5 ± 1.0	79.3 ± 3.0	43.3 ± 1.7
PMSF	95.1 ± 3.1	94.2 ± 0.0	106.5 ± 7.4	90.4 ± 0.7
DTT	107.2 ± 3.1	169.6 ± 8.4	113.1 ± 12.7	140.1 ± 4.0

ND: not detected. The compounds were dissolved in 0.05 M sodium acetate buffer pH 5.5 and the activity was assayed with 1% (w/v) birchwood xylan in 0.05 M sodium acetate buffer pH 5.5 at 50°C.

Table 3
Kinetics of purified Xyl I and Xyl II from *T. inhamatum*.

Parameter	Xyl I		Xyl II	
	OSX	BWX	OSX	BWX
V_{max} ($U \cdot mg\ prot^{-1}$)	2680.2	462.2	4553.7	1972.7
K_m ($mg \cdot mL^{-1}$)	14.5	1.6	10.7	4.0
K_{cat} (s^{-1})	436.0	210.5	1354.7	586.9
K_{cat}/K_m ($s^{-1} \cdot mM^{-1}$)	7.6	19.9	19.1	21.7

OSX: oat spelts xylan; BWX: birchwood xylan. The activity was assayed in 0.05 M sodium acetate buffer pH 5.5 at 50°C.

relative mobility to xylose [28], the two spots with lower mobility than xylobiose corresponded to xylotriose and xylotetrose.

4. Conclusions

This manuscript presents the first report about the purification and properties of two xylanases from *T. inhamatum* by a simple and inexpensive procedure. These enzymes were stable over a wide range of pH and the optimal conditions for their activities were around 50°C and pH 5.0 very similar to each other and also to the characteristics observed for the crude enzyme [21]. These two xylanases appear to be differentially modified products from the same gene because they have similar hydrolytic and physico-chemical properties, and differential glycosylation may explain the differences in molecular masses, the capacity to bind to DEAE-anion exchanger and thermal stabilities. The glycosylation explains some cases, but do not completely elucidate the functional and genetic basis for the multiplicity of these enzymes. Furthermore, a comparison of amino acid compositions indicates that three xylanases from *Trichoderma harzianum* E58 are products of distinct genes [35]. The results indicate possible employment of such enzymes in some industrial processes, which require activity in acid conditions, wide-ranging pH stability, such as for animal feed, or juice and wine industries.

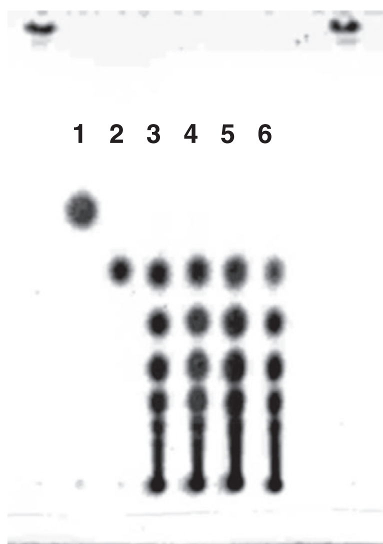

Fig. 7. Thin-layer chromatography of the hydrolysis products of oat spelts xylan by Xyl I from *T. inhamatum*. Row: 1 xylose. Row 2: xylobiose. Rows 3, 4, 5 and 6: 10 min, 30 min, 2 h and 17 h of hydrolysis, respectively.

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Conflict of interest

The authors state not having any conflict of interest in the publication of this article.

References

[1] Scheller HV, Hemicelluloses Ulvskov P. Annu Rev Plant Biol 2010;61:263–89. http://dx.doi.org/10.1146/annurev-arplant-042809-112315.
[2] Wong KKY, Tan LUL, Saddler JN. Multiplicity of β-1,4-xylanase in microorganisms: Functions and applications. Microbiol Rev 1988;52:305–17.
[3] Dodd D, Cann IKO. Enzymatic deconstruction of xylan for biofuel production. Glob Chang Biol Bioenergy 2009;1:2–17. http://dx.doi.org/10.1111/j.1757-1707.2009.01004.x.
[4] Puls J, Schuseil J. Chemistry of hemicellulose: Relationship between hemicellulose structure and enzymes required for hydrolysis. In: Coughlan MP, Hazlewood GP, editors. Hemicellulose and Hemicellulases. London: Portland Press; 1993. p. 1–27.
[5] Biely P. Microbial xylanolytic systems. Trends Biotechnol 1985;3:286–90. http://dx.doi.org/10.1016/0167-7799(85)90004-6.
[6] Polizeli MLT, Rizzati ACS, Monti R, Terenzi HF, Jorge JA, Amorim DS. Xylanases from fungi: Properties and industrial applications. Appl Microbiol Biotechnol 2005;67: 577–91. http://dx.doi.org/10.1007/s00253-005-1904-7.
[7] Techapun C, Poosaran N, Watanabe M, Sasaki K. Thermostable and alkaline-tolerant microbial cellulose-free xylanases produced from agricultural wastes and the properties required for use in pulp bleaching bioprocess: A review. Process Biochem 2003;38:1327–40. http://dx.doi.org/10.1016/S0032-9592(02)00331-X.
[8] Dhiman SS, Sharma J, Buttan B. Industrial application and future prospects of microbial xylanases: A review. BioResources 2008;3:1377–402.
[9] Deutschmann R, Dekker RFH. From plant biomass to bio-based chemicals: Latest developments in xylan research. Biotechnol Adv 2012;30:1627–40. http://dx.doi.org/10.1016/j.biotechadv.2012.07.001.
[10] Van den Brink J, de Vries RP. Fungal enzyme sets for plant polysaccharide degradation. Appl Microbiol Biotechnol 2011;91:1477–92. http://dx.doi.org/10.1007/s00253-011-3473-2.
[11] Wong KKY, Saddler JN. Trichoderma xylanases, their properties and application. Crit Rev Biotechnol 1992;12:413–35. http://dx.doi.org/10.3109/07388559209114234.
[12] Peterson R, Nevalainen H. Trichoderma reesei RUT-C30 — Thirty years of strain improvement. Microbiology 2012;158:58–68. http://dx.doi.org/10.1099/mic.0.054031-0.
[13] Ike M, Jeung-yil P, Tabuse M, Tokuyasu K. Controlled preparation of cellulases with xylanolytic enzymes from *Trichoderma reesei* (*Hypocrea jecorina*) by continuous-feed cultivation using soluble sugars. Biosci Biotechnol Biochem 2013;77:161–6. http://dx.doi.org/10.1271/bbb.120696.
[14] Silveira FQP, Sousa MV, Ricart CAO, Milagres AMF, Medeiros CL, Filho EXF. A new xylanase from a *Trichoderma harzianum* strain. J Ind Microbiol Biotechnol 1999;23: 682–5. http://dx.doi.org/10.1038/sj.jim.2900682.
[15] Chen LL, Zhang M, Zhang DH, Chen XL, Sun CY, Zhou BC, et al. Purification and enzymatic characterization of two β-endoxylanases from *Trichoderma* sp. K9301 and their actions in xylooligosaccharide production. Bioresour Technol 2009;100: 5230–6. http://dx.doi.org/10.1016/j.biortech.2009.05.038.
[16] Zhou P, Zhu H, Yan Q, Katrolia P, Jiang Z. Purification and properties of a psychrotrophic *Trichoderma* sp. xylanase and its gene sequence. Appl Biochem Biotechnol 2011;164:944–56. http://dx.doi.org/10.1007/s12010-011-9186-2.
[17] Min SY, Kim BG, Lee C, Hur H-G, Ahn JH. Purification, characterization and cDNA cloning of xylanase from fungus *Trichoderma* strain SY. J Microbiol Biotechnol 2002;12:890–4.
[18] Ahmed S, Aslam N, Latif F, Rajoka MI, Jamil A. Molecular cloning of cellulase genes from *Trichoderma harzianum*. Front Nat Prod Chem 2005;1:73–5. http://dx.doi.org/10.2174/1574089054583669.
[19] He J, Yu B, Zhang K, Ding X, Chen D. Expression of endo-1,4-beta-xylanase from *Trichoderma reesei* in *Pichia pastoris* and functional characterization of the produced enzyme. BMC Biotechnol 2009;9:56. http://dx.doi.org/10.1186/1472-6750-9-56.
[20] Tauk-Tornisielo SM, Garlipp A, Ruegger M, Attili DS, Malagutti E. Soilborne filamentous fungi in Brazil. J Basic Microbiol 2005;45:72–82. http://dx.doi.org/10.1002/jobm.200410418.
[21] Silva LAO, Carmona EC. Production and characterization of cellulase-free xylanase from *Trichoderma inhamatum*. Appl Biochem Biotechnol 2008;150:117–25. http://dx.doi.org/10.1007/s12010-008-8296-y.

[22] Vogel HJ. A convenient growth medium for *Neurospora* (Medium N). Microb Genet Bull 1956;13:42–3.
[23] Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 1959;31:426–8. http://dx.doi.org/10.1021/ac60147a030.
[24] Lowry OH, Rosebrough NF, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265–75.
[25] DuBois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. Anal Chem 1956;28:350–6. http://dx.doi.org/10.1021/ac60111a017.
[26] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5. http://dx.doi.org/10.1038/227680a0.
[27] Lineweaver H, Burk D. The determination of the enzyme dissociation constants. J Am Chem Soc 1934;56:658–66. http://dx.doi.org/10.1021/ja01318a036.
[28] Fontana JD, Gebara M, Blumel M, Schneider H, Mackenzie CR, Johnson HK. A-4-*o*-methyl-D-glucuronidase component of xylanolytic complexes. Methods Enzymol 1988;160:560–71. http://dx.doi.org/10.1016/0076-6879(88)60169-8.
[29] Törrönen A, Mach RL, Messner R, Gonzalez R, Kalkkinen N, Harkki A, et al. The two major xylanases from *Trichoderma reesei*: Characterization of both enzymes and genes. Bio/Technology 1992;10:1461–5.
[30] Lappalainen A, Siika-Aho M, Kalkkinen N, Fegerström R, Tenkanen M. Endoxylanase II from *Trichoderma reesei* has several isoforms with different isoelectric points. Biotechnol Appl Biochem 2000;31:61–8. http://dx.doi.org/10.1042/BA19990066.
[31] Bastawde KB. Xylan structure, microbial xylanases, and their mode of action. World J Microbiol Biotechnol 1992;8:353–68. http://dx.doi.org/10.1007/BF01198746.
[32] Liab K, Azadi P, Collins R, Tolan J, Kim JS, Eriksson KEL. Relationships between activities of xylanases and xylan structures. Enzyme Microb Technol 2000;27:89–94. http://dx.doi.org/10.1016/S0141-0229(00)00190-3.
[33] Fialho MB, Carmona EC. Purification and characterization of xylanases from *Aspergillus giganteus*. Folia Microbiol 2004;49:13–8. http://dx.doi.org/10.1007/BF02931639.
[34] Ryan SE, Nolan K, Thompson R, Gubitz GM, Savage AV, Tuohy MG. Purification and characterization of a new low molecular weight endoxylanase from *Penicillium capsulatum*. Enzyme Microb Technol 2003;33:775–85. http://dx.doi.org/10.1016/S0141-0229(03)00176-5.
[35] Wong KKY, Tan LUL, Saddler JN, Yaguchi M. Purification of a third distinct xylanase from the xylanolytic system of *Trichoderma harzianum*. Can J Microbiol 1986;32:570–6. http://dx.doi.org/10.1139/m86-106.