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# Two β-xylanases from *Aspergillus terreus*: Characterization and influence of phenolic compounds on xylanase activity

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ABSTRACT

Sugarcane bagasse was used as an inexpensive alternative carbon source for production of β-xylanases from *Aspergillus terreus*. The induction profile showed that the xylanase activity was detected from the 6th day of cultivation period. Two low molecular weight enzymes, named Xyl T1 and Xyl T2 were purified to apparent homogeneity by ultrafiltration, gel filtration and ion exchange chromatographies and presented molecular masses of 24.3and 23.60 kDa, as determined by SDS–PAGE, respectively. Xyl T1 showed highest activity at 50 °C and pH 6.0, while Xyl T2 was most active at 45 °C and pH 5.0. Mass spectrometry analysis of trypsin digested Xyl T1 and Xyl T2 showed two different fingerprinting spectra, indicating that they are distinct enzymes. Both enzymes were specific for xylan as substrate. Xyl T1 was inhibited in greater or lesser degree by phenolic compounds, while Xyl T2 was very resistant to the inhibitory effect of all phenolic compounds tested. The apparent km values of Xyl T2, using birchwood xylan as substrate, decreased in the presence of six phenolic compounds. Both enzymes were inhibited by N-bromosuccinimide and $Hg^{2+}$ and activated by $Mn^{2+}$. Incubation of Xyl T1 and Xyl T2 with L-cysteine increased their half-lives up to 14 and 24 h at 50 °C, respectively. Atomic force microscopy showed a bimodal size distribution of globular particles for both enzymes, indicating that Xyl T1 is larger than Xyl T2.



## 1. Introduction

Agro-industrial residues are a rich source of lignocellulosic material, whose structure is chiefly represented by the physico-chemical interactions of cellulose with hemicellulose and lignin (Andreaus et al., 2008). Within this context, the agro-industrial residues represent an important alternative source for the microbial growth and production of industrial enzymes (Yeoman et al., 2010).

Recalcitrance to saccharification is a major limitation for the conversion of lignocellulosic biomass to valuable end products. An intricate arrangement between polysaccharides of the cell wall matrix, hereafter called holocellulose, proteins and lignin makes the cell wall structure a challenge for carbohydrase and ligninase enzyme systems from different sources (Duarte et al., 2012a; Saha, 2003). The pretreatment of lignocellulosic biomass is crucial before the enzymatic hydrolysis of the holocellulose structure of the plant cell wall. Chemical, physical, and morphological lignocellulose features are changed during pretreatment and generally make the biomass more accessible to enzymatic saccharification. Various pretreatment options are available to fractionate, solubilize, hydrolyze, and separate cellulose, hemicellulose, and lignin components. Liquid hot water, steam explosion and dilute acid pretreatments generate soluble inhibitors which hamper enzymatic hydrolysis as well as fermentation of sugars to ethanol (Kim et al., 2011). Among those inhibitors are phenols, including lignin degradation products, hydroxynamic acid derivatives, tannins and gallic acid (Ximenes et al., 2011). Although the inhibitory or deactivator effects of phenolic compounds on cellulases and hemicellulases have been well documented in the literature, little is known about the nature of interactions or the inhibitory mechanisms (Boukari et al., 2011; Ximenes et al., 2011).

Given that xylan represents the major hemicellulosic component from lignocellulosic biomass, β-xylanases are produced by a wide variety of fungal (Polizeli et al., 2005). Since the last century, β-xylanases have received much attention because of their practical applications in various agro-industrial processes, such as

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efficient conversion of hemicellulosic biomass to fuels and chemicals, delignification of paper pulp, digestibility enhancement of animal feedstock, clarification of juices, and improvement in the consistency of beer (Beg et al., 2001; Polizeli et al., 2005; Saha, 2003).

In a previous work, Siqueira et al., (2010) reported that among twenty-one fungal species tested the highest yield of β-xylanase activity was produced by *Aspergillus terreus* when grown in liquid media containing agro-industrial residue as the carbon source. Our study focused on the use of sugarcane bagasse (SCB) as a carbon source for *A. terreus* cultivation and the subsequent production, purification and characterization of two β-xylanases with different behaviors in the presence of phenolic compounds.

## 2. Materials and methods

### 2.1. Residue pretreatment

SCB was autoclaved at 121 °C for 2 h and thoroughly washed with tap water. After autoclaving, it was dried at 65 °C for 48 h and then grounded to form a relative homogeneous blend. A fine powder was obtained and used as the carbon source.

### 2.2. Organism and enzyme production

*A. terreus* was isolated from the natural composting of textile wastes and maintained in the fungus culture collection of the Enzymology Laboratory, University of Brasilia, Brazil and maintained in PDA medium (2% potato broth, 2% dextrose and 2% agar). The spore concentration was determined by counting under a microscope with a Neubauer chamber, and was adjusted with sterile saline solution (0.9%) to a final concentration of $10^8$ spores/mL. For xylanase production, an aliquot (5.0 mL) of spore suspension was inoculated into Erlenmeyer flasks. The cultures were incubated at 28 °C with constant agitation at 120 rpm for 6 days in a liquid medium containing (*w*/*v*) 0.7% $KH_2PO_4$, 0.2% $K_2HPO_4$, 0.05% $MgSO_4{\cdot}7H_2O$ and 0.16% $(NH_4)_2SO_4$ at pH 7.0, with 1% (*w*/*v*) of SCB as the carbon source. After the culture had grown, the medium was filtered through a Büchner funnel with filter paper (Whatman No. 1), and stored at 4 °C. The resulting filtrate, hereafter called crude extract, was used as source of xylanase. For β-xylanase induction, aliquots were harvested every 24 h during 28 days and used to estimate the enzyme activity and protein concentration.

### 2.3. Enzyme assays

The β-xylanase activity was determined by mixing 5 μL of enzyme solution with 10 μL of birchwood xylan (10 mg/mL) in 50 mM sodium phosphate buffer, pH 7.0 at 50 °C for 30 min. The release of the reducing sugar was measured using the DNS method (Miller, 1959), and the β-xylanase activity was expressed as μmol of reducing sugar released per min per milliliter (IU/mL). Xylose was used as standard. The activity against filter paper was measured as previously described (Ghose, 1987). Endoglucanase (CMCase), pectinase and mannanase activities were determined as reported elsewhere (Duarte et al., 2012b). For the experiments involving *p*-nitrophenyl glycosides, β-xylosidase, β-glucosidase, β-mannosidase and α-arabinofuranosidase activities were determined with the substrates *p*-nitrophenyl-β-D-xylopyranoside (*p*NPX), *p*-nitrophenyl-β-D-glucopyranoside (ρNPG), *p*-nitrophenyl-β-D-mannopyranoside (ρNPM) and *p*-nitrophenyl-α-arabinofuranoside (ρNPA), respectively as previously described (Ximenes et al., 1996). Protein concentration was measured by the method of Bradford (Bradford, 1976), using bovine serum albumin as standard.

### 2.4. Enzyme purification

All chromatographic steps were performed at room temperature. The crude extract was concentrated approximately 10-fold by ultrafiltration using an Amicon System (Amicon Inc., Beverly, MA, 01915, USA) with a membrane having a cutoff point of 10 kDa. Aliquots of the retentate (100 mL) and ultrafiltrate (900 mL) were fractionated by gel filtration chromatography on a Sephadex G-50 (GE Healthcare Life Sciences) column (51.0 × 3.6 cm) equilibrated with 50 mM of sodium phosphate buffer, pH 7.0. Fractions (5 mL) were eluted at a flow rate of 20 mL/h, and those corresponding to β-xylanase activity were pooled and loaded onto a DEAE-Sephacel (GE Healthcare Life Sciences) column (8.0 × 3.0 cm) equilibrated with the same buffer described above. Fractions of 5 mL were collected at a flow rate of 30 mL/h by washing the column with buffer followed by a linear gradient of NaCl (0–1.0 mol/L), pH 7.0. Fractions corresponding to β-xylanase activity were pooled and stored at 4 °C for further characterization. The purified β-xylanases from retentate and ultrafiltrate were denominated Xyl T1 and Xyl T2, respectively.

### 2.5. Enzyme characterization

For the kinetics experiments, soluble and insoluble birchwood xylans were prepared as described by Filho et al. (1993). The substrates were used in a concentration range of 0.33–13.3 mg/mL. km and Vmax values were estimated from the Michaelis–Menten equation with a non-linear regression data analysis program (Enzfitter) (Leatherbarrow, 1999). The effects of temperature, pH, ions, EDTA and other agents on Xyl T1 and Xyl T2 activities were determined as described by Duarte et al. (2012b). Each assay described above was repeated at least three times. Appropriated controls were used for each experiment and the standard deviation was less than 10% of the mean.

### 2.6. Electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out using a 12% gel (Laemmli, 1970). After electrophoresis, the protein bands were silver stained by the method of (Blum et al., 1987). Replicate denaturing electrophoretic gel, containing 0.1% birchwood xylan, was submitted to zymogram analysis (Sunna et al., 1997). The molecular masses of Xyl T1 and Xyl T2 were estimated by SDS–PAGE using low molecular mass markers from GE Healthcare Life Sciences.

### 2.7. Mass spectrometry

Proteins of interest were separated by SDS–PAGE in gel digested (Shevchenko et al., 2006) and analyzed using matrix-assisted laser desorption/ionization (MALDI)-time-of-flight (TOF)/TOF MS. After trypsin digestion the resulting peptides were desalted and concentrated in a reverse phase column (POROS R2) and then eluted using a matrix mixture (1:1) consisting of 5 μg/μL 2,5-dihydroxybenzoic acid (DHB; diluted in ACN 90% and formic acid 0,5%) and 5 μg/μL α-cyano-4-hydroxycinnamic acid (CHCA; diluted in ACN 90% and TFA 0,01%). Samples were then loaded onto a 600-nm AnchorChipTM target plate (Bruker Daltonics, Karlsruhe, Germany) and allowed to dry completely and subjected to MS analysis. MS analysis was performed using a MALDI-TOF/TOF mass spectrometer (Autoflex II; BrukerDaltonics). Mass spectra were processed using Flex Analysis 2.2 (BrukerDaltronics).

### 2.8. Deactivation by phenolic compounds

The effect of phenolic compounds in the Xyl T1 and Xyl T2 activities was measured by pre-incubating the enzyme samples with ferulic acid, ρ-coumaric acid, vanillin, cinnamic acid, 4-hydroxybenzoic acid and tannic acid at concentration of 1 mg/mL. The incubation was at 25 °C during 7 days. Aliquots were withdrawals at intervals to measure β-xylanase activity.

### 2.9. Statistical analysis

The experiments of deactivation by phenolic compounds, the effects of ions, EDTA and other agents were analyzed with the software Statistica 8.0. Data were submitted to factorial ANOVA and post hoc test LSD Fisher Pairwise Comparison, with significance $P < 0.05$.

### 2.10. Atomic force microscopy (AFM)

Xyl T1 and Xyl T2 were analyzed by atomic force microscopy (AFM) to investigate their topographical features. In order to remove salts, Xyl T1 and Xyl T2 were previously washed against Milli-Q water. A piece disk of freshly cleaved mica was attached onto an iron stub with a double-sided adhesive tape; then about 3 μL was dropped onto the mica surface; finally, the samples were air-dried. In order to remove the salt, Xyl T1 and Xyl T2 were previously dialyzed against Milli-Q water. Samples were analyzed using a commercial AFM instrument (SPM-9600 Shimadzu, Japan), operating in force constant dynamic mode with image acquisition height (topography) and phase (viscoelasticity). Images were obtained in air, at approximately 20 °C and 20% relative humidity. Scanned areas were perfect squares from 5 μm × 5 μm with scan rate of 1 Hz, applying no more than 500 pN of constant force. Trace and retrace scanning procedures were performed in order to prove that the samples were not modified during scanning steps. All AFM images contained 512 × 512 pixels, and the raw data images were processed and rendered with the aid of the software SPM-9600 *off-line* (version 3.304, Shimadzu, Japan). Statistical analyses were performed with the software OriginPro (version 8.0, Originlab, USA).

## 3. Results and discussion

### 3.1. Enzyme production and purification

The induction profile during growth of *A. terreus* on SCB showed that β-xylanase activity increased steadily and reached the highest value (0.096 IU/mL) on the 6th day of growth (data not shown). The growth profile was accompanied by several peaks of protein with the highest value (0.148 mg/mL) on the 11th and 12th days of growth. This protein profile probably includes other proteins, in addition to β-xylanases, which are simultaneously produced and may be involved in the complex process of SCB degradation. Therefore, based on the growth curve of the fungus, we established 6 days for fungal growth in liquid medium containing SCB.

Ultrafiltration procedure showed that most of the β-xylanase activity was found in the retentate, while a small percentage was detected in the ultrafiltrate. The β-xylanase activity of the retentate and ultrafiltrate were 0.715 and 0.329 IU/mL, respectively. In addition, the amount of protein of retentate and ultrafiltrate were 5.6 and 0.22 mg/mL, respectively. The ability of β-xylanase to change their conformation and pass through membranes with cutoff point of 10 kDa could be explored for applications in pulp bleaching, whereas it involves the enzyme ability to pass through the small pores and thus to penetrate hemicellulose–lignin–cellulose matrix in the cellulose pulp (Beg et al., 2001). The elution profile of retentate on Sephadex G-50 resulted in the separation of two peaks of β-xylanase activity (data not shown). The first peak of β-xylanase was eluted in the single protein peak and further purified by chromatography on DEAE-Sephacel. In this purification procedure, Xyl T1 was eluted in the pre-gradient wash fractions. The ultrafiltrate profile on Sephadex G-50 showed a single peak of β-xylanase activity that eluted before a major peak of protein. This enzyme peak was further fractionated by anion-exchange chromatography. The elution profile on DEAE-Sephacel resulted in only one pool of β-xylanase activity (Xyl T2), which also eluted before application of salt gradient. The purification steps of Xyl T1 and Xyl T2 are summarized in Table 1. β-Xylanases from fungi have been purified to apparent homogeneity by different chromatographic procedures, including gel filtration and ion-exchange with different recovery and purification yield (Sá-Pereira et al., 2003). Xyl T1 and Xyl T2 had purification yields of 5.7 and 74.7%, respectively. The low purification yield of Xyl T1 is consistent with other studies involving β-xylanase purification in which the recovery was low (Sá-Pereira et al., 2003). Since β-xylanases act synergistically for the complete hydrolysis of xylan and other form of β-xylanase (Xyl T2) was detected in the ultrafiltrate, the yield and fold purification of Xyl T1 were probably underestimated. SDS–PAGE of Xyl T1 and Xyl T2 showed single bands with molecular masses of 24.3 and 23.60 kDa, respectively (data not shown). The low molecular mass values of Xyl T1 and Xyl T2 is characteristic of xylanases from GH 11 family (Paés et al., 2012). Higher molecular mass values of 33 and 67 kDa were determined for β-xylanases from *A. terreus* strains BCC129 and $S_7$, respectively (Chantasingh et al., 2006; Pal et al., 2006). Zymogram analysis of the Xyl T1 and Xyl T2 samples showed a single band of β-xylanase activity coincident with that staining for protein (data not shown). Although the two enzymes bands migrate very close to each other, the peptide mass fingerprint (PMF) analysis by mass spectrometry after the digestion of the xylanases with trypsin showed a completely different profile of the spectra, which indicates that Xyl T1 and Xyl T2 are two different enzymes. Unfortunately, the analysis by fragmentation of the peptides obtained (MS/MS) was not able to identify any specific protein. Chidi et al. (2008) reported that MALDI-TOF/TOF and LC mass spectroscopy gave 8 peptide ions whose sequence alignments showed that the β-xylanase produced by *A. terreus* strain UL 4209 has homology with those of other *Aspergillus* strains such as *A. terreus* and *A. versicolor*.

### 3.2. Characterization of Xyl T1 and Xyl T2

Xyl T1 was most active at 50 °C, retaining at least 68% of its maximal activity at temperatures interval of 40–55 °C. On the other hand, Xyl T2 activity was highest at 45 °C, and retained 86% of its maximal activity at the same temperature range. However, at temperature above 50 °C Xyl T1 and Xyl T2 showed a gradual decline of their activities with a decrease of 75% at 70 °C. Xyl T1 displayed a high activity at pH range of 4.0–7.5, being most active at pH 6.0. Contrary to Xyl T1, Xyl T2 had maximal activity at pH 5.0 and maintained at least 50% of its activity at pH interval of 4.0–7.0. With respect to thermostability, Xyl T1 and Xyl T2 retained 50% of their activities up to 2 and 3.5 h of incubation at 50 °C, respectively. Incubation of Xyl T1 and Xyl T2 with L-cysteine at 50 °C increased their half-lives up to 14 and 24 h, respectively. The observed protective effect of cysteine could be due to the presence of a reducing environment that would keep L-cysteine residues monothiols in a reduced state, avoiding the formation of disulfide bridges that could impair the native tertiary structure of the protein.

Xyl T1 and Xyl T2 were completely inactivated when incubated with $Hg^{2+}$ at the concentration of 10 mM, suggesting the presence of thiol groups in the catalytic site of the enzyme (Table 2). The ions $Ag^{+}$ (concentration of 10 mM) and $K^{+}$ (1 and 10 mM

**Table 1**
Summary of purification steps of Xyl T1 and Xyl T2 β-xylanases from *A. terreus*.

| Purification steps | Total protein (mg) | Total activity (IU) | Specific activity (IU/mg) | Purification(fold) | Yield (%) |
|---|---|---|---|---|---|
| Crude extract | 27.315 | 2.616 | 0.096 | 1.000 | 100.00 |
| *Xyl T1* | | | | | |
| Retentate | 6.5 | 0.286 | 0.044 | 0.460 | 10.93 |
| Sephadex G-50 | 2.95 | 0.221 | 0.075 | 0.783 | 8.46 |
| DEAE-Sephacel | 0.094 | 0.149 | 1.578 | 16.48 | 5.7 |
| *Xyl T2* | | | | | |
| Ultrafiltrate | 4.975 | 1.184 | 0.238 | 2.486 | 45.27 |
| Sephadex G-50 | 1.683 | 1.953 | 1.160 | 12.114 | 74.65 |
| DEAE-Sephacel | 3.290 | 1.954 | 0.594 | 6.201 | 74.7 |

**Table 2**
The effect of metallic ions and EDTA on the β-xylanase activity of purified Xyl T1 and Xyl T2.

| | Xyl T1 Relative activity (%) | | Xyl T2 Relative activity (%) | |
|---|---|---|---|---|
| | 1 mM | 10 mM | 1 mM | 10 mM |
| Control | 100.00 ± 0.006 | 100.00 ± 0.02 | 100.00 ± 0.053 | 100.00 ± 0.030 |
| $MgSO_4$ | 97.40 ± 0.005 | 94.67 ± 0.009 | 107.77 ± 0.019 | 104.25 ± 0.011 |
| $MnCl_2$ | 110.02 ± 0.005 | 136.78 ± 0.008* | 115.24 ± 0.001* | 161.63 ± 0.020* |
| $AgNO_3$ | 101.71 ± 0.005 | 84.40 ± 0.018 | 118.68 ± 0.038 | 69.85 ± 0.028* |
| $FeSO_4$ | 102.30 ± 0.015 | 96.92 ± 0.012 | 71.99 ± 0.024* | 79.98 ± 0.042* |
| $CoCl_2$ | 115.95 ± 0.003* | 95.04 ± 0.010 | 95.06 ± 0.049 | 81.87 ± 0.002 |
| $FeCl_3$ | 103.14 ± 0.008 | 72.39 ± 0.013* | 85.99 ± 0.022 | 86.61 ± 0.007* |
| $CuSO_4$ | 85.25 ± 0.005* | 86.52 ± 0.027 | 84.96 ± 0.047 | 75.95 ± 0.022* |
| EDTA | 97.71 ± 0.010 | 91.55 ± 0.010 | 101.06 ± 0.003 | 93.45 ± 0.008 |
| $CaCl_2$ | 98.10 ± 0.010 | 86.75 ± 0.022 | 91.059 ± 0.019 | 95.20 ± 0.006 |
| $MgCl_2$ | 102.23 ± 0.003 | 68.90 ± 0.013* | 81.03 ± 0.012 | 98.94 ± 0.015 |
| $ZnCl_2$ | 98.21 ± 0.007 | 92.29 ± 0.029 | 94.33 ± 0.029 | 100.15 ± 0.003 |
| $ZnSO_4$ | 98.21 ± 0.009 | 100.20 ± 0.005 | 89.61 ± 0.033 | 98.94 ± 0.003 |
| $HgCl_2$ | 104.2 ± 0.001* | 00.00 ± 0.001* | 86.72 ± 0.034 | 00.00 ± 0.020* |
| KCl | 79.09 ± 0.01* | 69.26 ± 0.006* | 79.54 ± 0.024* | 79.70 ± 0.05* |
| NaCl | 98.12 ± 0.01 | 96.20 ± 0.012 | 91.29 ± 0.009 | 98.85 ± 0.040 |
| $CuCl_2$ | 98.13 ± 0.01 | 101.01 ± 0.005 | 86.96 ± 0.052 | 102.38 ± 0.040 |

* Indicates statistical difference in LSD Fisher test.

concentrations) also had an inhibitory effect on both enzymes. On the other hand, Xyl T1 and Xyl T2 had an increase of 36% and 61%, in their activities, respectively when incubated with10 mM $Mn^{2+}$, indicating its possible role as cofactor in the enzyme-substrate reaction (Ghanem et al., 2000). The other metallic ions, including $Mg^{2+}$, $Ca^{2+}$, $Zn^{2+}$, $Cu^{2+}$ and $Na^{+}$, and EDTA did not affect the β-xylanase activity of Xyl T1 and Xyl T2.

Xyl T1 and Xyl T2 were strongly inhibited by NBS, which is primarily involved in oxidation of tryptophan residues (Table 3). A weak inhibition of both enzymes activities was noted in the presence of the alkylating reagent iodoacetamide, suggesting that these enzymes require thiol groups for their stabilities (Teixeira et al., 2010). On the other hand, an increase of 15 and 11% on the activities of Xyl T1 and Xyl T2, respectively, was noted in the presence of L-tryptophan, which in turn might be involved in the active site, participating in binding and/or hydrolysis of the substrate and in maintaining the integrity of active site. Only Xyl T2 was activated by L-cysteine, suggesting involvement in hydrogen-bonding with the substrate, enzyme folding and the formation of covalent glycosyl–xylan intermediate. Although there are reports in the literature of an overestimation of xylose concentration from xylan enzymatic hydrolysis during the DNS reaction in the presence of 20 mM cysteine (Teixeira et al., 2012), we did not detect the interference of L-cysteine in the DNS assay. The treatment of Xyl T2 with DTNB activated the enzyme activity, also suggesting an influence of L-cysteine in the catalysis of xylan. For other compounds containing thiol groups, it was observed activation (β-mercaptoethanol) and inhibition (DTT) of Xyl T1. Inactivation by DEPC (Xyl T1 and Xyl T2) and EDC (Xyl T2) suggest the involvement of histidine and carboxyl groups. Xyl T1 and Xyl T2 were slightly activated by DTP and SDS, respectively.

**Table 3**
Effect of modifying reagents and amino acids on the β-xylanase activity of Xyl T1 and XylT2.

| Reagent or amino acid | Concentration (mM) | Relative activity (%) – Xyl T1 | Relative activity (%) – Xyl T2 |
|---|---|---|---|
| $H_2O$ Control | – | 100.00 ± 0.011 | 100.00 ± 0.010 |
| 4% Ethanol Control | – | 100.00 ± 0.020 | 100.00 ± 0.014 |
| DTNB[a] | 2 | 88.13 ± 0.014* | 117.16 ± 0.006* |
| DTT | 20 | 75.78 ± 0.008* | 109.11 ± 0.007 |
| L-Cysteine | 20 | 96.71 ± 0.003 | 149.86 ± 0.012* |
| NBS | 1 | 48.61 ± 0.027* | 11.083 ± 0.086* |
| DTP[a] | 2 | 118.66 ± 0.008* | 72.09 ± 0.037* |
| L-Tryptophan | 5 | 114.69 ± 0.006 | 110.68 ± 0.012* |
| Iodoacetamide | 5 | 89.40 ± 0.020* | 85.28 ± 0.006 |
| DEPC | 5 | 81.58 ± 0.012* | 70.07 ± 0.102* |
| EDC | 5 | 100.09 ± 0.006 | 65.44 ± 0.043* |
| β-mercaptoethanol | 5 | 122.92 ± 0.005* | 105.83 ± 0.040 |
| SDS | 20 | 96.35 ± 0.014 | 114.00 ± 0.014 |

[a] Compounds diluted in 4% ethanol.
* Indicates statistical difference in LSD Fisher test.

The purified enzymes were devoid of measurable FPase activity. The residual activity was less than 10% for CMC and mannan as the substrates. Both enzymes acted mainly on soluble birchwood, oat spelt and beechwood xylans (Table 4). The strict substrate specificity of Xyl T1 and Xyl T2 suggests that these enzymes are members of GH11 family (Paés et al., 2012). This substrate specificity also indicates that both enzymes have strong potential for use in pulp bleaching, whereas application of β-xylanases in the pulp and paper industry requires a cellulase-free activity (Beg et al., 2001; Christov et al., 1999; Sandrim et al., 2005). Xyl T1 and Xyl T2 were completely inactive toward the *p*-nitrophenyl glycoside substrates.

### 3.3. Deactivation by phenolic compounds

The inhibitory effect of phenols on Xyl T1 and Xyl T2 was evaluated by incubating these enzymes with phenolic compounds and their residual xylanase activity after incubation was measured. The concentration of phenolic compounds (1 mg/mL) had no interference in the DNS assay. In a previous experiment, two higher concentrations (5 mg/mL and 10 mg/mL) of these phenolic compounds were found to interfere in the DNS assay, increasing the absorbance values (data not shown).

Xyl T1 activity was strongly affected by all phenolic compounds. 4-hydroxybenzoic acid highly affects Xyl T1, inhibiting about 40% of its initial activity on contact with the enzyme. Cinnamic acid and vanillin completely deactivate the enzyme with 72 h of incubation (Fig. 1 A). Ximenes et al. (2010) showed that the activities of cellulase and β-glucosidase of *A. niger* were affected by the same

**Table 4**
Substrate specificity of Xyl T1 and Xyl T2.

| Substrate | Main chain linkage | Activity (IU/mL) Xyl T1 | Activity (IU/mL) Xyl T2 |
|---|---|---|---|
| Birchwood xylan (S) | β-1,4 | 0.400 ± 0.006 | 0.360 ± 0.011 |
| Birchwood xylan (I) | β-1,4 | 0.120 ± 0.050 | 0.140 ± 0.018 |
| Oat spelt xylan (S) | β-1,4 | 0.259 ± 0.008 | 0.225 ± 0.028 |
| Oat spelt xylan (I) | β-1,4 | 0.073 ± 0.019 | 0.064 ± 0.085 |
| Beechwood xylan (S) | β-1,4 | 0.253 ± 0.038 | 0.208 ± 0.029 |
| Beechwood xylan (I) | β-1,4 | 0.087 ± 0.015 | 0.096 ± 0.023 |
| Filter paper | β-1,4 | 0.000 ± 0.001 | 0.000 ± 0.001 |
| CM-cellulose | β-1,4 | 0.038 ± 0.006 | 0.035 ± 0.003 |
| Mannan | β-1,4 | 0.046 ± 0.015 | 0.075 ± 0.022 |
| Pectin | α-1,4 | 0.042 ± 0.004 | 0.046 ± 0.008 |
| ρNPX | ρNP-β-1,4 | 0.000 ± 0.000 | 0.000 ± 0.005 |
| ρNPA | ρNP-α-1,4 | 0.000 ± 0.003 | 0.000 ± 0.03 |
| ρNPG | ρNP-β-1,4 | 0.000 ± 0.000 | 0.000 ± 0.005 |
| ρNPM | ρNP-β-1,4 | 0.000 ± 0.003 | 0.000 ± 0.000 |

(S) Soluble (I) Insoluble.

phenolic compounds used in our study. Another study showed that guaiacol (2-methoxyphenol) and caffeic acid (3,4-dihydroxycinnamic acid) significantly inhibited the activity of a xylanase contained in crude extracts from *A. japonicas* (Sharma et al., 1985). Similarly, compounds leaching from lignin in pulping liquors produced significant inhibition of a β-xylanase from *Trichoderma harzianum* (Senior et al., 1990). It is expected that the deactivation of the enzyme increases with the increasing time of exposure to the deactivating conditions, and this effect was noted with almost all phenolic compounds tested against Xyl T1. The half-life of Xyl T1 at room temperature was 5 days. However, in the presence of ferulic and ρ-coumaric acids its half-life was reduced to 48 and 72 h, respectively. The greatest effect was detected with cinnamic acid and vanillin, which decreased Xyl T1 half-life to 15 min. A non-competitive multi-site inhibition mechanism of phenolic compounds, including cinnamic acid; ρ-coumaric acid; caffeic acid; ferulic acid; and 3,4,5-trimethoxy-cinnamic acid, was proposed for the enzyme activity of a purified β-xylanase (Tx-Xyl) from *Thermobacillus xylanilyticus* (Boukari et al., 2011). These interactions are likely to involve residues located at the surface of Tx-Xyl, indicating that the inhibitory effects of phenolic compounds on Tx-Xyl activity are most likely brought about by conformational alterations of the enzyme inducing steric inactivation.

Xyl T2 was very resistant to all phenolic compounds tested (Fig. 1B). In the absence of phenolic compounds (control), the enzyme showed no loss of activity even after 7 days of incubation at room temperature. Cinnamic acid, the only compound with inhibitory action on Xyl T2, had a deactivator effect with 7 days of incubation. It was reported by Kaya et al. (2000) that the addition of vanillic acid increased the rate of enzymatic hydrolysis of xylan at low concentrations (up to 0.05%), while at higher concentrations they began to inhibit the enzyme activity of a commercial xylanase preparation (Irgazyme-40S, Ciba-Geigy Corporation, Greensboro, NC). Vanillic acid increased the enzymatic hydrolysis rate of xylan by 50%, as compared to the control value. Hydrolysis of xylan was improved by the addition of vanillin and guaiacol. Addition of increasing concentrations of vanillin and guaiacol caused more than 15% and 175% increasing in the enzymatic hydrolysis rate of xylan, respectively.

Xyl T2 has great potential application in the second-generation bioethanol production, since it was resistant to six important phenolic compounds for 7 days. Phenolic compounds can have varying effects on xylanase activity, increasing the activity under certain conditions. Therefore, activity of xylanase and the presence of lignin degradation products are not likely to be correlated in a simple way (Kaya et al., 2000).

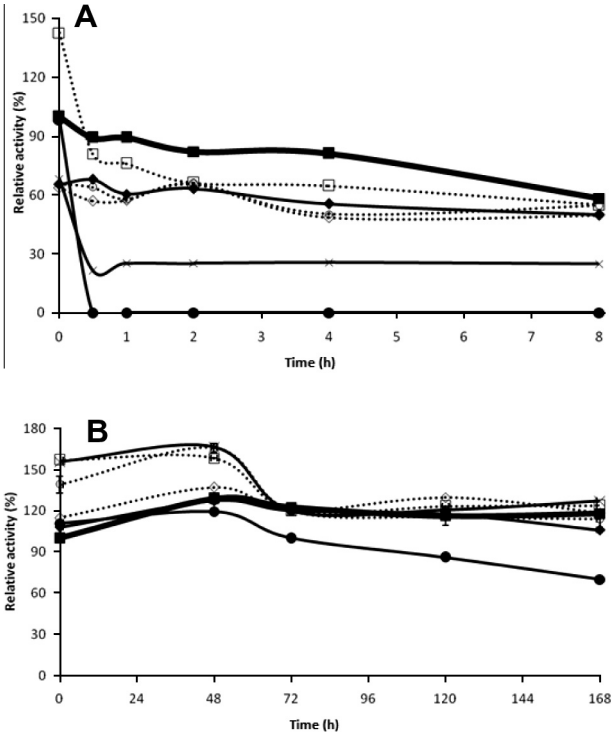


**Fig. 1.** Incubation of phenolic compounds with Xyl T1 (A) and Xyl T2 (B). Control (■), Ferulic acid (○), ρ-Coumaric acid (□), Cinnamic acid (●), Vanillin (×), 4-Hydroxybenzoic acid (◇), Tannic acid (♦).

In the absence of phenolic compounds, Xyl T1 had close km values over insoluble and soluble xylans (Table 5). In comparison with the km values of Xyl T1, Xyl T2 presented lower affinity for xylan, with a preference for insoluble xylan as substrate (Table 6). This might suggest a steric hindrance due to the presence of substituents in soluble xylan. According to some reports, xylanases from GH11 family do not tolerate high substitutions on the xylan backbone (Biely et al., 1997; Paés et al., 2012). The km values described in this study are lower than that reported for a xylanase (16.7 mg/mL) from *A. terreus* (Ghanem et al., 2000). The km values of Xyl T1 were reduced in the presence of ferulic acid, ρ-coumaric acid and vanillin. In an opposite way, cinnamic acid, tanic acid and 4-hydroxybenzoic acid decreased the affinity of Xyl T1 for soluble and insoluble xylans. Although it was detected a decrease in the Vmax values of Xyl T2, it is noteworthy that the incubation of Xyl T2 with all phenolic compounds decreased substantially the km values, suggesting that the phenolic compounds enhanced the affinity of Xyl T2 for its substrate. Further work is needed to clarify the effect of these phenolic compounds on Xyl T1 and Xyl T2 activities. Some reports describe that interactions of phenolic compounds with cellulase have been correlated to changes in the structure of the enzyme (Boukari et al., 2011; Tian et al., 2013). These interactions may include both hydrophobic aromatic ring stacking (between phenolic compounds and tryptophanyl side chains) and/or hydrogen interactions between their (COOH, OH) functional groups and the basic amino acid residues. Fluorescence spectroscopy studies (Tian et al., 2013) showed that the covalent binding of ferulic acid and ρ-coumaric acid changed the structure and hydrophobic environment of cellulase, affecting its activity. Circular dichroism analysis also revealed that the addition of the phenolic acids significantly decreased α-helix content but increased β-sheet and random coil contents of a commercial preparation of cellulase (Tian et al., 2013). As a result, the enzyme structure became more flexible and its affinity for the substrate increased.

**Table 5**
Kinetic parameters of Xyl T1 in the absence and presence of phenolic compounds.

| | Soluble xylan | | Insoluble xylan | |
|---|---|---|---|---|
| | km | Vmax | km | Vmax |
| Control | 0.421 ± 0.035 | 0.147 ± 0.001 | 0.465 ± 0.037 | 0.078 ± 0.003 |
| Ferulic acid | 0.216 ± 0.036 | 0.121 ± 0.024 | 0.901 ± 0.015 | 0.152 ± 0.001 |
| ρ-Coumaric acid | 0.301 ± 0.083 | 0.225 ± 0.009 | 0.244 ± 0.001 | 0.167 ± 0.001 |
| Cinnamic acid | 0.453 ± 0.030 | 0.140 ± 0.003 | 0.465 ± 0.043 | 0.116 ± 0.004 |
| Vanillin | 0.166 ± 0.031 | 0.233 ± 0.003 | 0.457 ± 0.052 | 0.203 ± 0.004 |
| 4-Hydroxy-benzoic acid | 0.905 ± 0.001 | 0.184 ± 0.001 | 0.476 ± 0.012 | 0.138 ± 0.001 |
| Tannic acid | 0.645 ± 0.089 | 0.247 ± 0.008 | 0.305 ± 0.013 | 0.258 ± 0.002 |

**Table 6**
Kinetic parameters of Xyl T2 in the absence and presence of phenolic compounds.

| | Soluble xylan | | Insoluble xylan | |
|---|---|---|---|---|
| | km | Vmax | km | Vmax |
| Control | 13.03 ± 0.278 | 1.016 ± 0.013 | 10.90 ± 0.290 | 1.20 ± 0.190 |
| Ferulic acid | 0.258 ± 0.001 | 0.094 ± 0.001 | 0.195 ± 0.005 | 0.094 ± 0.004 |
| ρ-Coumaric acid | 0.668 ± 0.048 | 0.186 ± 0.003 | 0.080 ± 0.003 | 0.123 ± 0.001 |
| Cinnamic acid | 0.202 ± 0.020 | 0.119 ± 0.001 | 0.115 ± 0.022 | 0.078 ± 0.001 |
| Vanillin | 0.352 ± 0.026 | 0.127 ± 0.001 | 0.141 ± 0.030 | 0.093 ± 0.002 |
| 4-Hydroxy-benzoic acid | 1.004 ± 0.003 | 0.121 ± 0.000 | 0.839 ± 0.292 | 0.105 ± 0.008 |
| Tannic acid | 0.141 ± 0.001 | 0.110 ± 0.001 | 0.278 ± 0.044 | 0.166 ± 0.004 |

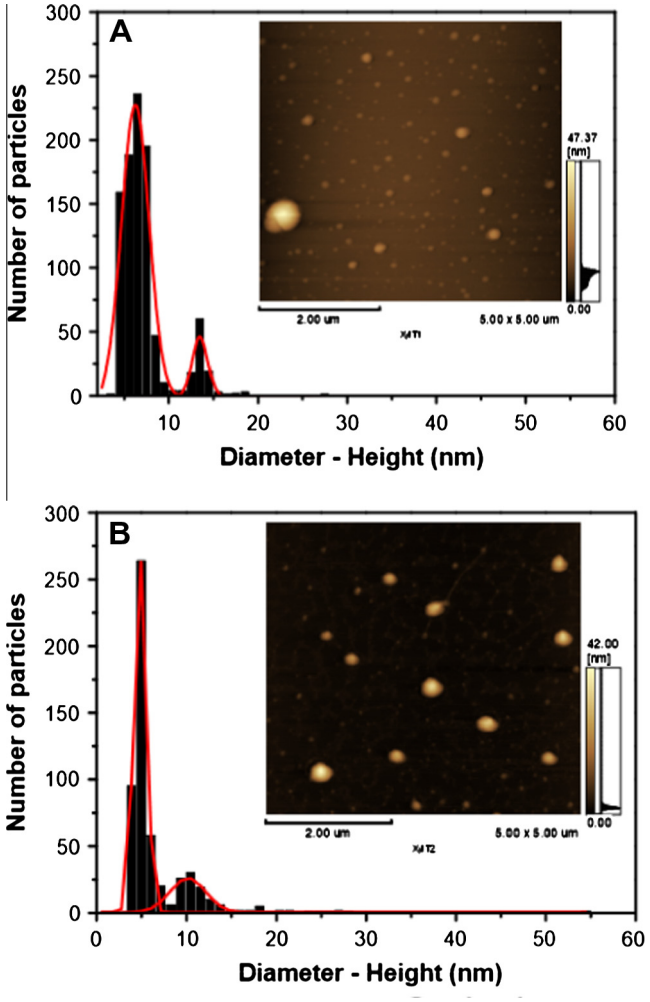


**Fig. 2.** AFM images of purified Xyl T1 (A) and Xyl T2 (B) deposited onto mica surface at a concentration of 10 μg/mL and histogram of height distribution of both enzymes.

### *3.4. Atomic Force Microscopy (AFM)*

The analysis of Xyl T1 and Xyl T2 by AFM was designed to obtain information about the possible topographical differences between both enzymes in nanoscale. Both enzymes showed bimodal distribution. In the case of Xyl T1, 89.5% of the particles were observed in the first Gaussian with height mean of 6.26 nm and 10.5% were in the second class, with an average size of 13.5 nm (Fig 2 A). Concerning to Xyl T2, 80.1% of the particles were in the first class, with mean height of 4.82 nm while 19.9% were in the second class, with average size of 10.26 nm. Xyl T2 showed more abundance of higher height (second class) structures than XylT1, moreover, Xyl T2 is smaller than Xyl T1, as demonstrated in Fig. 2 B. Some few still larger structures were seen in Xyl T2, however these structures can be liable for the protection of the active site of Xyl T2, conferring resistance to phenolic compounds. Alternatively, Xyl T2 could have a missing segment, which would preclude the binding of these compounds to the enzyme structure. Furthermore, interactions of phenolic compounds with tyrosine and tryptophan residues would be responsible for changes in Xyl T2 structure.

## 4. Conclusion

In conclusion, two low-molecular-weight xylanases produced by *A. terreus* were purified and characterized. These xylanases hydrolyzed preferentially xylan as the substrate, being attractive enzymes for potential future applications in the pulp and paper industries, since industry application requires a cellulase-free activity. Xyl T2 showed a great resistance to six different phenolic compounds, which is of high industrial interest in biofuel production, since these compounds are formed during lignocellulosic biomass pre-treatment. A reduction in km of Xyl T2 was also observed in the presence of all phenolic compounds, apparently increasing the affinity between this enzyme and the xylan. AFM showed topographical differences between Xyl T1 and Xyl T2. Based on the molecular mass and catalytic properties, Xyl T1 and Xyl T2 are suggested to belong to family GH1. Further studies will investigate the structural features of Xyl T1 and Xyl T2. The use of circular

dichroism and fluorescence spectroscopy as tools to understand the interactions between the enzymes and phenolic compounds must be considered in a near future.

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