.African Journal of Biotechnology Vol. 11(32), pp. 8076-8084, 19 April, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.2686
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Purification and characterization of an extracellular xylanase produced by the endophytic fungus, *Aspergillus terreus*, grown in submerged fermentation

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Accepted 14 March, 2012

Aspergillus terreus **produced high levels of a thermotolerant extracellular xylanase and showed low cellulase activity when cultured at 30°C for 48 h, in liquid medium supplemented with wheat bran as carbon source. Xylanase was purified 45-fold to homogeneity with a recovery yield of 67% by carboxymethyl (CM)-cellulose chromatography. The enzyme, a glycoprotein with 33% of carbohydrate content, appeared as a single protein band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel with a molecular mass corresponding to 23 kDa. Optimal temperature and pH were 55°C and 4.5, respectively. The enzyme was thermotolerant at 45 and 50°C, with a half-life of 55 and 36 min, respectively. The K_m was calculated as 22 mg/ml and V_{max} as 625 mg/ml of protein using birchwood xylan as substrate. Metal ions, such as Ag^+, Cu^{+2}, Fe^{+2}, Hg^+ and Zn^{+2} strongly inhibited xylanase, whereas K^+ and Mn^{+2} resulted in activation. Xylanase hydrolyzed birchwood xylan and oat-spelt xylan, mostly yielding xylooligosaccharides, suggest that it is an endoxylanase (EC. 3.2.1.37).**

Key words: *Aspergillus terreus*, endoxylanase, thermostability.

INTRODUCTION

Xylan is the principal hemicellulose and is the second most abundant renewable carbon source found in nature and can be degraded to useful end products (Polizeli et al., 2005; Squina et al., 2009). Xylan's basic molecular structure is a linear backbone that comprised of β-1,4-linked D-xylopyranose residues that, according to their origin, may be substituted by acetyl or arabinosyl groups and glucopyranosyl residues (Squina et al., 2009). The complete hydrolysis of xylan requires the combined action of various enzymes as endo-β-1,4-xylanase (EC 3.2.1.8), exoxylanase (β-D-xylan xylohydrolase) and β-xylosidase (EC 3.2.1.37) which are the most characterized in the literature (Yang et al., 2006; Fang et al., 2010). Xylanases randomly hydrolyze the β-1,4-glycosidic bonds of xylan, producing several xylo-oligomers, whereas β-xylosidases release xylosyl residues by endwise attack of xylooligosaccharides (Fang et al., 2007).

The interest in xylanases and xylan has increased markedly because of their industrial applications, particularly, their potential biotechnological use in various processes, such as kraft pulp bleaching, food processing (including bread-making and beer and juice clarification), bioconversion of lignocelluloses residues into fermentable sugars for ethanol production and other useful substances without modifying the physical and chemical properties of products (Canju et al., 1989; Carmona et al.,1998; Lu et al., 2008; Jiang et al., 2010). In the last decade, one of the most studied and promising application of xylanases is in kraft pulp biobleaching (Sandrim et al., 2005; Savitha et al., 2007; Betini et al., 2009; Peixoto-Nogueira et al., 2009; Michelin et al., 2010; Yeasmin et al., 2011), because of their ability to improve

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pulp fibrillation and water retention, reduce beating time in virgin pulps, retard recycled fiber breakdown and promote selective removal of xylans from dissolving pulps. Other promising area is the application of xylanases in animal feed intending to improve digestibility (Facchini et al., 2011).

Although, many reports are available about thermostable xylanase production by mesophilic microorganisms, few studies have addressed the production of hydrolytic enzymes by endophytic fungi, such as *Aspergillus terreus*. Preliminary tests performed in our laboratory showed that *A. terreus* is an excellent producer of xylanase when compared with other filamentous fungi tested. In this context, the present study focuses on the production and purification of *A. terreus* and describes some biochemical properties of an extracellular xylanase produced by this mesophilic and endophytic filamentous fungus. This xylanase exhibited remarkable thermostability, which improves its biotechnological potential.

MATERIALS AND METHODS

Microorganism and culture

A. terreus strain was isolated from *Memora peregrina*, a native plant from Cerrado biome in Mato Grosso do Sul State, Southwestern Brazil. The strain was identified at Universidade Federal de Pernambuco. The fungus was maintained in the laboratory at 30°C on slants of solid potato dextrose agar (PDA) medium. Approximately 10^7 conidia from 7-day-old cultures were inoculated into 125 ml Erlenmeyer flasks containing 25 ml medium described by Rizzatti et al. (2001) using 1% (w/v) of the desired carbon sources (1% of glucose, sucrose, or agroindustrial residues such as rice straw, sugarcane bagasse, wheat bran or corncob), at pH 6.0.

The cultures were incubated under orbital agitation (100 rpm) or stationary conditions, at 30°C, during different periods (24 to 144 h). The medium was subsequently vacuum-filtered using filter paper (Whatman) and the crude filtrate was used for the study of the extracellular enzyme.

Enzyme assay

Xylanase activity was assayed at 55°C using citrate-phosphate buffer (McIlvaine, 1921) containing 1.0% (w/v) of birchwood xylan as substrate. The amount of reducing sugar released was determined using the 3,5-dinitrosalicylic acid (DNS) method proposed by Miller (1959), employing xylose (Sigma) as the standard. One unit of enzyme activity was defined as the amount of enzyme which releases 1 µmol of xylose per minute under assay condition. Specific activities were expressed as U/mg of protein. Protein concentrations were determined using the Lowry method (Lowry et al., 1951), with bovine serum albumin (BSA) as the standard.

Purification of extracellular xylanase

A. terreus was grown during 48 h, at liquid medium. The crude extract was obtained by vacuum filtration and it was equilibrated with 50 mM sodium acetate buffer at pH 4.5, and was loaded on a carboxymethyl (CM)-cellulose column (2.0 × 6.5 cm) equilibrated under the same conditions. Bound xylanase was eluted at a flow rate of 50 ml/h with a linear 0 to 1 M sodium chloride gradient in the same buffer. Fractions exhibiting high absorbance were pooled, dialyzed against distilled water and used for biochemical characterization. The purified enzyme was stored at -20°C.

Gel electrophoresis (SDS-PAGE)

Protein homogeneity and enzyme molecular mass were evaluated using 10% of SDS-PAGE as described by Laemmli (1970). The gels were stained with Coomassie Brilliant Blue R-250. The molecular standard employed was a low-molecular-weight calibration kit for SDS electrophoresis (Amersham) that is composed of bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.2 kDa).

Biochemical characterization of purified xylanase

The suitable pH value for xylanase activity was investigated using citrate-phosphate buffer in the pH range from 3.5 to 8.0. Optimal temperature for xylanase activity was determined by incubating the enzyme at different temperatures (35 to 70°C). To determine thermal stability, the enzyme was incubated at different temperatures and during different periods (10 to 60 min). The residual xylanase activity was measured according to the standard assay method. The influence of metal ions (5 mM) on xylanase activity was investigated under the standard assay conditions comparing with a control without metal ions. Total neutral carbohydrate was quantified by the phenol-sulfuric acid method proposed by Dubois et al. (1956) using xylose as the standard. To determine enzyme substrate specificity, purified xylanase was incubated with 1% of different substrates in McIlvaine buffer at pH 4.5 at 50°C. The amount of reducing sugars produced was determined using the DNS method as described above.

Identification of hydrolysis products by thin layer chromatography (TLC)

Xylan hydrolysis products were analyzed using TLC on G-60 silica gel plates. The reaction mixtures were incubated at 55°C for 30, 60 and 420 min. The reactions were stopped by boiling the samples for 5 min. Thereafter, 3 µl were applied on TLC which was developed in two runs with a mixture of ethyl acetate: acetic acid: formic acid: water (9:3:1:4 v/v/v). The plates were prayed with a mixture of orcinol 0.2% (w/v) in sulfuric acid: methanol (1:9 v/v) and incubated at 100°C until the spots were visualized.

Kinetic parameters

The kinetic parameters (Michaelis-Menten constant, K_m, and maximum reaction velocity, V_{max}) were determined by incubating the enzyme under optimal conditions of temperature and pH with different concentrations of birchwood xylan (0.225 to 38.25 mg/ml) and estimating their values using linear regression from double-reciprocal plots, according to Lineweaver and Burk (1934).

Reproducibility of results

All results are expressed as the means of at least three independent experiments [±standard deviation (SD)].

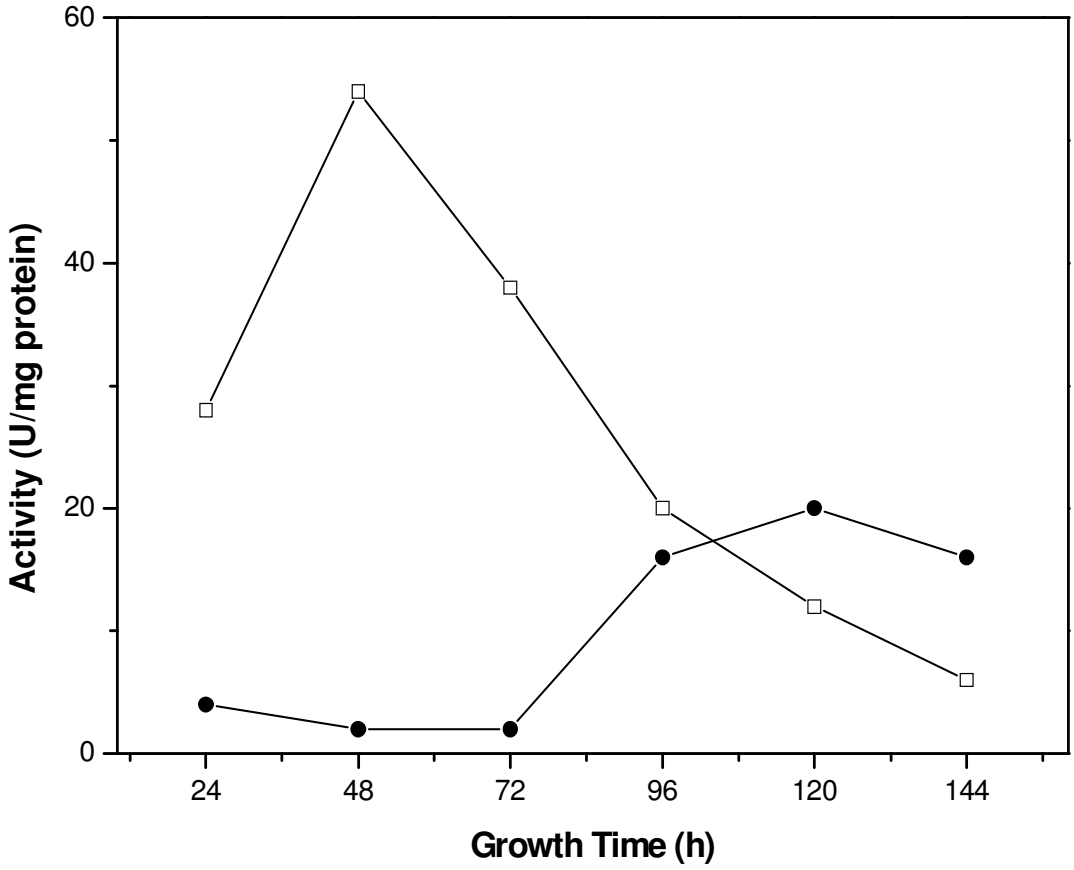

Figure 1. Time-course of extracellular xylanase production by *A. terreus* in SR liquid medium. □, under agitation (100 rpm); ●, stationary condition. Values are means ± SD of three experiments.

RESULTS AND DISCUSSION

Time course for enzyme production

Nutritional and environmental factors, such as the type of carbon source, period of cultivation, use (or not) of agitation, etc., may affect enzyme synthesis and production by fungi. The time course for xylanase production by *A. terreus* is presented in Figure 1. Under agitation, the enzyme production rapidly increased within 48 h of incubation and the maximum activity (54 U/mg of protein) was observed. Growth over 48 h on agitation condition had a sharp decline in enzyme activity. Under static conditions, enzyme activity also increased during the experiment, but not as much as under agitation.

Xylanase induction by agroindustrial residues

Xylanase production by *A. terreus* was induced using different lignocellulosic materials (Table 1). It produces high specific activity and total units (U) when the fungus was grown on wheat bran, sugarcane bagasse or corncob as the main carbon source. Growth on glucose, sucrose or rice straw resulted in a lower production. Corncob, wheat bran and xylan are known to be good microorganism xylanase inducers (Pal and Khanum, 2010). Among all the agroindustrial residues tested, wheat bran was found to induce the production of xylanase with the highest activity and hence, it was selected for further experiments. The choice of wheat bran was important, because it is an agroindustrial residue, cheaper in comparison to xylan and when used for xylanase production, it may result in a final product with a lower price (Peixoto-Nogueira et al., 2009). The use of wheat bran has attracted considerable attention as a substrate for solid-state fermentation for xylanases and other enzymes (Peixoto-Nogueira et al., 2008).

Xylanase purification from *A. terreus*

An extracellular xylanase was purified from the supernatant obtained from *A. terreus* culture grown on wheat bran at 30°C for 48 h. The purification procedure is summarized in Table 2 and the chromatographic profile is

Table 1. Effect of carbon source on xylanase production by *A. terreus*.

Carbon source	Total activity (total U)	Total protein (total mg)	Specific activity (U/mg of protein)
Sugarcane bagasse	2223 ±0.52	43 ±0.76	51 ±0.21
Rice straw	838 ±0.42	39 ±0.6	21 ±0.15
Wheat bran	5124 ±0.32	79 ±0.55	65 ±0.20
Corncob	3152 ±0.2	58 ±0.36	54 ±0.53
Sucrose	1108 ±0.51	26 ±0.25	42 ±0.21
Glucose	774 ±0.52	34 ±0.1	23 ±0.36

A. terreus was grown under agitation (100 rpm), using SR (Rizzatti et al., 2001) liquid medium, at 30°C for 48 h. Values are means ±SD of three experiments.

Table 2. Purification steps of an extracellular xylanase from *A. terreus*.

Parameter	Total activity (U)[a]	Total protein (mg)[b]	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	133.5	860	0.155	100	1
CM-celullose	90.0	13	6.92	67	45

[a]Activity measured at 55°C, using 1% of Birchwood xylan in McIlvaine buffer (pH 4.5). [b]Protein content estimated using BSA as the standard.

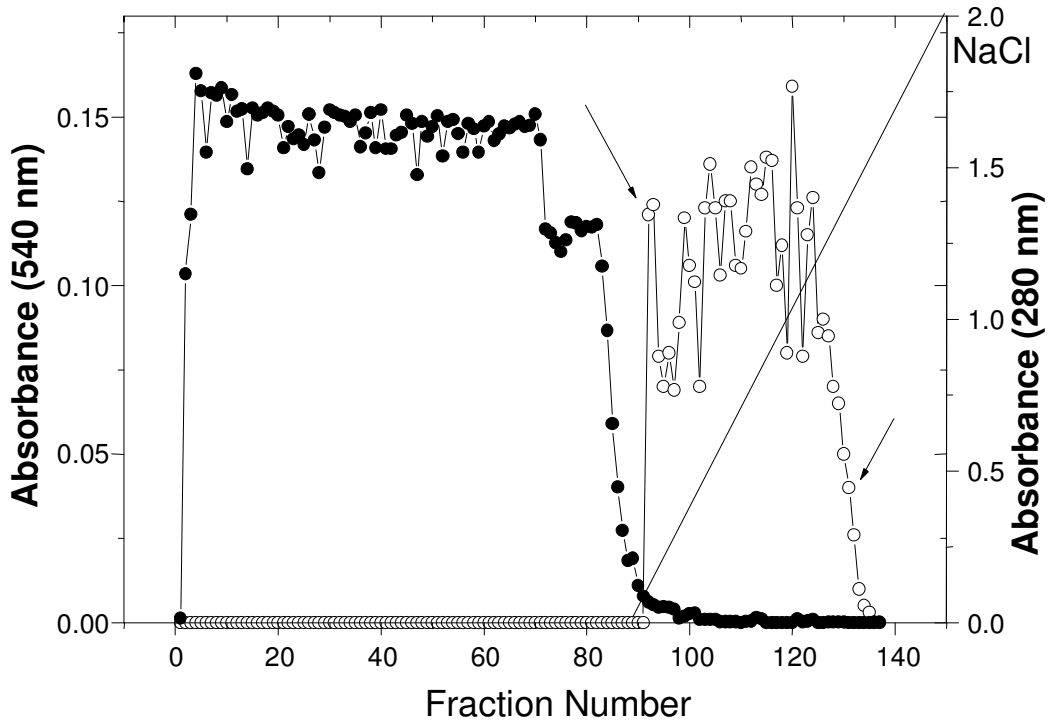

Figure 2. CM-cellulose ion exchange chromatography of a xylanase produced by *A. terreus*. Arrows indicate pooled fractions exhibiting xylanase activity. The column was equilibrated with 50 mM acetate buffer at pH 4.5 and eluted with a linear salt gradient (0 to 1 M) in the same buffer. Flow rate and fraction size were 50 ml/h and 5.0 ml, respectively. ●, A280; ○ xylanase activity; —, NaCl concentration.

presented in Figure 2. The enzyme was purified to 45-fold with a 67% recovery yield. The enzyme appeared as a single protein band on SDS-PAGE gel, with a molecular mass, approximately corresponding to 23 kDa (Figure 3). Similar results were presented by *Chaetomium thermophilum* var. *coprophile* (26 kDa) (Canju et al.,

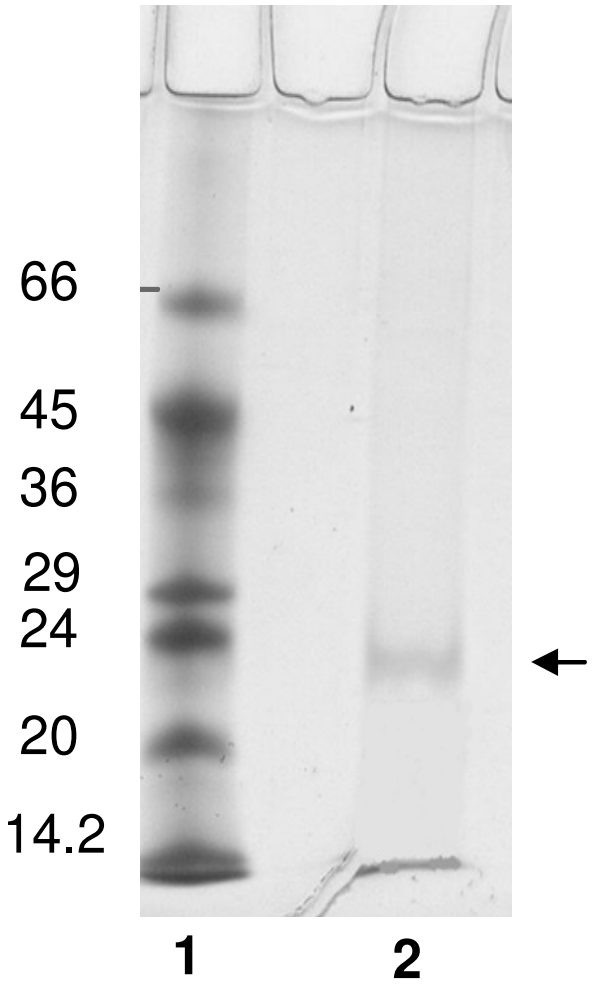

Figure 3. SDS-PAGE 10% of the purified xylanase, stained with Coomassie Brilliant Blue R-250. Lane 1, molecular weight standards (kDa); lane 2, 14 µg of purified extracellular xylanase.

1989), *Chaetomium cellulolyticum* (Baraznenok et al., 1999) and *Paecilomyces thermophila* (Li et al., 2006) (25 kDa), but were higher than xylanases produced by *Acrophialophora nainiana* (Salles et al., 2000) and *Streptomyces matensis* (Yan et al., 2009), which corresponded to 22.2 kDa and 21.1 kDa, respectively.

A. terreus xylanase obtained in this investigation had 33% of carbohydrate content, lower than that previously measured for a xylanase from *Penicillium sclerotioum* (14.8%) (Knob and Carmona, 2010), but higher than xylanases from *P. thermophila* (21%) (Yang et al., 2006) and *Avicularia versicolor* (14%) (Carmona et al., 2005). Glycosylation is a common feature among extracellular fungal xylanases (Wong et al., 1988) and provides a post-translational modification mechanism that modulates secretion of enzymes. It has been shown that xylanase glycosylation may contribute to the stability of protein conformation, thus increasing enzymatic activity.

Effect of pH, temperature and thermostability on xylanase activity

Some properties of the extracellular xylanase from *A. terreus* were studied using xylan from birchwood as substrate in McIlvaine buffer in different pH (3.5 to 8.0). The purified xylanase was most active at pH 4.5, but substantial activity was detected in the pH range from 5.0 to 6.5 (Figure 4A). Optimal activity in acid conditions makes xylanase attractive for industrial applications, for instance, in the feed and food industries.

Analyzing the effect of temperature on xylanase activity, it is possible to observe that at 55°C, the optimal activity values were obtained (Figure 4B). This optimal temperature value was comparable to that observed for xylanases from a number of mesophilic and thermophilic fungi, ranging from 35 to 60°C (Ryan et al., 2003; Sandrim et al., 2005) (Figure 5).

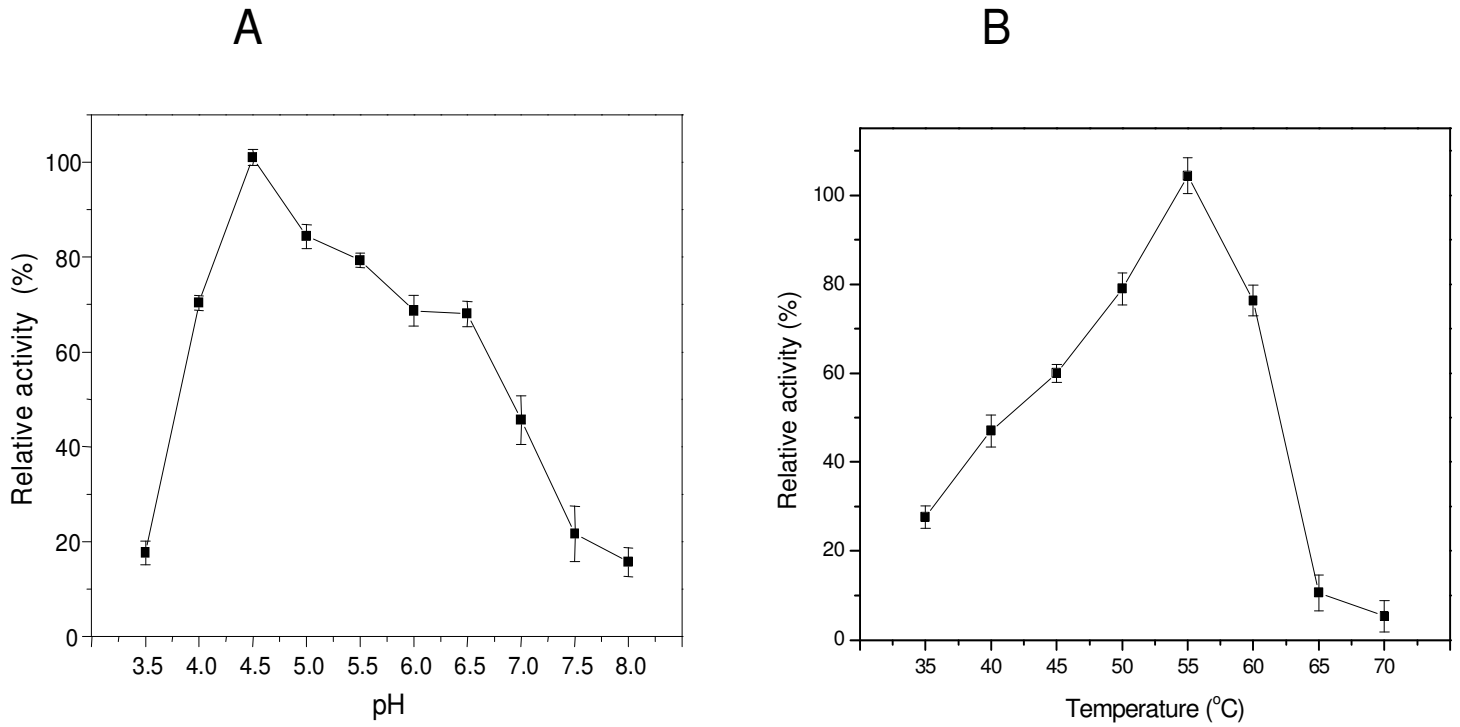

Figure 4. Effect of pH (A) and reaction temperature (B) on the activity of a purified xylanase from *A. terreus*. Values are means ± SD of three experiments.

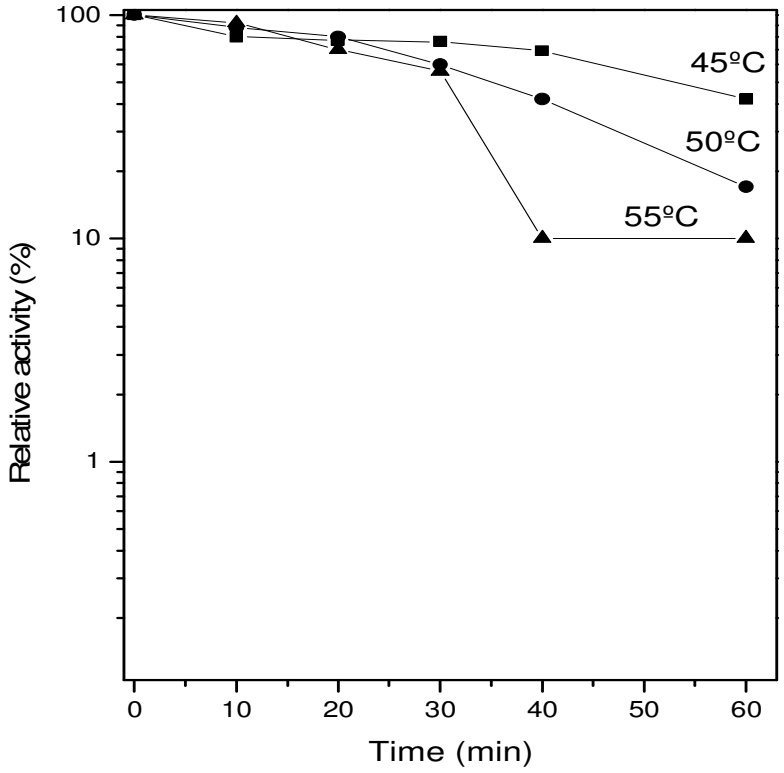

Figure 5. Thermal stability for activity of a xylanase from *A. terreus*. The purified xylanase was incubated without substrate at 45°C (■), 50°C (●) and 55°C (▲).

Table 3. Effect of metal ions and EDTA on the relative activity of a purified xylanase from *A. terreus*.

Substance (5 mM)	Residual activity (%)[a]
Control	100 ±1.15
$AgNO_3$	ND
$AlCl_3$	88 ±2.08
$BaCl_2$	98 ±1.45
$BaSO_4$	69 ±2.72
$CaCl_2$	97 ±1.15
$CoCl_2$	81 ±1.20
$CuSO_4$	53 ±1.73
EDTA	40 ±1.85
$FeSO_4$	57 ±2.96
$HgCl_2$	40 ±1.45
KCl	134±1.35
$MgSO_4$	70 ±1.45
$MnCl_2$	119 ±2.38
NH_4Cl	87 ±2.08
$ZnCl_2$	40 ±2.33

Assays were performed in McIlvaine buffer pH 4.5, at 55ºC using 1% Birchwood xylan as the substrate. ND, No detected activity within 2 h of enzyme reaction. [a]Values are means ±SD of three experiments.

In analyzing the thermostability data, it was observed that the half-life ($T_{1/2}$) at 50°C was 36 min, but the enzyme retained 80% of its activity after 10 min at 45°C. Generally, enzymes that exhibit reaction temperature and thermal stability at high temperatures (above 40°C) are attractive for biotechnological applications in several industrial areas.

Effect of metal ions on xylanase activity

The influence of certain metal ions and ethylene-diaminetetraacetic acid (EDTA) on xylanase activity was investigated. Among all tested metal ions, only Ag^{+} completely inhibited enzyme activity, while Cu^{+2}, Fe^{+2}, Hg^{+2}, Mg^{+2} and Zn^{+2} reduced enzyme activity by 47, 43, 60, 30 and 60%, respectively (Table 3). On the other hand, K^{+} and Mn^{+2} ions slightly stimulated xylanase activity, indicating the potential roles of both metal ions as cofactors for enzyme-substrate reactions, in addition to a stabilizing effect on various other enzymes. Xylanase activity was strongly inhibited by Hg^{+2}, a strong inhibitor of most xylanases, including those produced by *Thermomyces lanuginosus* (Singh et al., 2003), *Aspergillus caespitosus* (Sandrim et al., 2005), *Syncephalestrum racemosum* Cohn (Sapre et al., 2005), *P. thermophila* (Li et al., 2006), and *Penicillium citrinum* (Dutta et al., 2007). EDTA, a metal chelator, decreased xylanase activity, indicating that metal ions are required for the activity of purified enzymes.

Table 4. Substrate specificity of a purified extracellular xylanase from *A. terreus.*

Substrate[a]	Relative activity (%)[b]
Birchwood xylan	100 (±0.5)
Oat-spelt xylan	72 (±0.3)
Pectin (from citrus fruits)	ND
Polygalacturonic acid	ND
Avicel	ND
CM-cellulose	ND
Starch	ND
Sucrose	ND

ND, No activity detected within 2 h of enzyme reaction. [a]All substrates were tested in McIlvaine buffer at pH 4.5 at 55°C. Artificial substrates and natural substrates were used at a final concentration of 10 mg/ml. [b]Values are mean ±SD of three experiments.

Substrate specificity and kinetic parameters

Purified xylanase was assayed for hydrolytic activity against a variety of natural substrates. The enzyme was highly specific towards the xylans tested (Table 4). Activity was high against soluble birchwood xylan (100%), but lower against oat-spelt xylan (72%). The enzyme did not interact with starch, sucrose, carboxy-methyl cellulose, pectin, polygalacturonic acid or microcrystalline cellulose. The effect of birchwood xylan concentration on xylanase activity was investigated in the range of 0.225 to 38.25 mg/ml per reaction mixture. The initial velocity of the reaction was measured as a function of substrate concentration and was plotted as a double reciprocal, in accordance with Lineweaver-Burk analysis (Figure 6). Plotting revealed a K_m value of 22 mg/ml and a V_{max} value of 625 U/mg of protein. Both K_m and V_{max} were in agreement with the values reported for other fungal xylanases, which range from 0.09 to 40.9 mg/ml for K_m and from 0.106 to 6300 µM/min/mg for V_{max} (Beg et al., 2001). In this study, K_m was similar to the value previously obtained for *Talaromyces thermophilus* (22.5 mg/ml) (Yang et al., 2006) and *P. sclerotiorum* (23.4 mg/ml) (Knob and Carmona, 2010), indicating that the investigated xylanase presented higher affinity for birchwood xylan and high catalytic efficiency to hydrolyze the substrate.

Hydrolysis pattern

To test whether the investigated xylanase was an endoxylanase, birchwood xylan was hydrolyzed by the purified enzyme and the hydrolysis products applied on

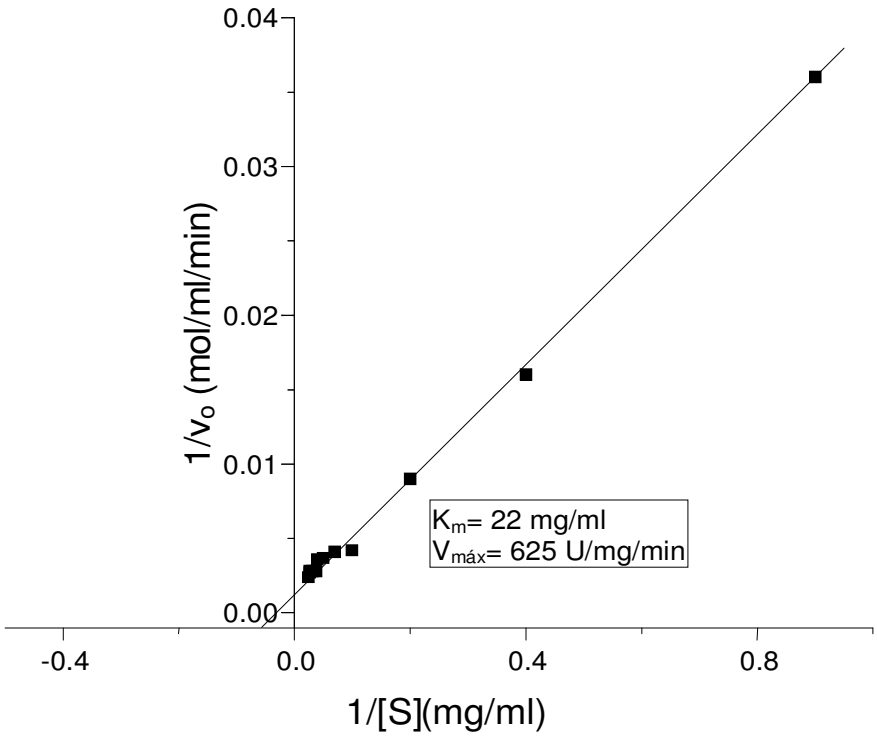

Figure 6. Enzyme activity was Measured in McIlvaine buffer at pH 4.5 at 55 ° C, using Xylan birchwood (0.225 to 38.25 mg / mL of protein) as the substrate.

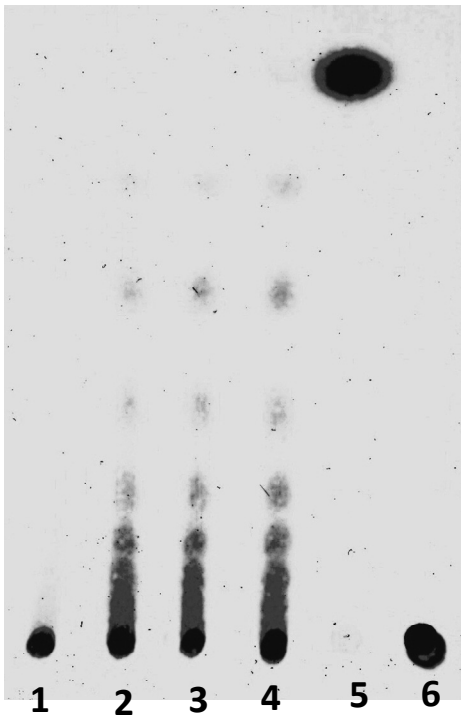

Figure 7. TLC of hydrolysis products of birchwood xylan upon reaction with a purified extracellular xylanase from *A. terreus*. Lanes 1 to 4, hydrolysis products using xylanase I after 0, 30, 60 and 420 min of incubation, respectively; lanes 5 and 6, hydrolysis products of the standards (xylose and xylan, respectively).

TLC (Figure 7). It was revealed that xylooligosaccharides as xylotriose, xylotetraose and larger ones were the principal hydrolysis products formed, especially after longer enzyme action (30, 60 and 408 min). Apparently, the enzyme released exclusively xylooligosaccharides, decreasing polymerization degree to xylotriose, a result that allows the classification of the enzyme as a β-D-1,4-xylan xylanohydrolase (E.C. 3.2.1.37), a typical endoxylanase.

ACKNOWLEDGEMENTS

The authors wish to thank CNPq (Brazil) and Fundect (Mato Grosso do Sul State, Brazil) for their financial support and for the scholarship awarded to the first author.

REFERENCES

Baraznenok VA, Becker EG, Ankudimova NV, Okunev NN (1999). Characterization of neutral xylanases from *Chaetomium cellulolyticum* and their biobleaching effect on eucalyptus pulp. Enzyme Microb. Technol. 25: 651-659.

Beg QK, Kapoor M, Mahajan L, Hoondal GS (2001). Microbial xylanases and their industrial applications: a review. Appl. Microbiol. Biot. 56: 326-338.

Betini JHA, Michelin M, Peixoto-Nogueira SC, Jorge JA, Terenzi H F, Polizeli MLTM (2009). Xylanases from *Aspergillus niger*, *Aspergillus niveus* and *Aspergillus ochraceus* produced under solid-state fermentation and their application in cellulose pulp bleaching.

Bioprocess Biosyst. Eng. 32(6): 819-824.
Canju RK, Vithayathil PJ, Murthy SK (1989). Purification and characterization of two xylanases from *Chaetomium thermophile* var. coprophile. Can. J. Microb. 35(9): 836-842.
Carmona E, Brochetto-Braga MR, Pizzirani-Kleiner AP, Jorge JA (1998). Purification and biochemical characterization of an endoxylanase from *Aspergillus versicolor*. FEMS Microbiol. Lett. 166: 311-315.
Carmona EC, Fialho MB, Buchgnani EB, Coelho GD, Brocheto-Braga MR, Jorge JA (2005). Production, purification and characterization of a minor form of xylanase from *Aspergillus versicolor*. Process Biochem. 40(1): 359-364.
Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956). Colorimetric methods for determination of sugars and related substances. Anal. Chem. 28(3): 350-356.
Dutta T, Sengupta R, Sahoo R, Sinha RS, Bhattacharjee A, Ghosh S (2007). A novel cellulase free alkaliphilic xylanase from alkali tolerant *Penicillium citrinum*: Production, purification and characterization. Lett. Appl. Microbiol. 44(2): 206-211.
Facchini FD, Vici AC, Reis VR, Jorge JA, Terenzi HF, Reis RA, Polizeli MLTM (2011). Production of fibrolytic enzymes by *Aspergillus japonicus* C03 using agro-industrial residues with potential application as additives in animal feed. Bioprocess Biosyst. Eng. 34(3): 347-355.
Fang HY, Chang SM, Hsieh MC, Fang TJ (2007). Production, optimization growth conditions and properties of the xylanase from *Aspergillus carneus* M34. J Mol. Catal B-Enzyme, 49(16): 36-42.
Fang TJ, Liao BC, Lee SC (2010). Enhanced production of xylanase by *Aspergillus carneus* M34 in solid-state fermentation with agricultural waste using statistical approach. New Biotechnol. 27(1): 25-32.
Jiang Z, Cong Q, Yan Q, Kumar N, Du X (2010). Characterisation of a thermostable xylanase from *Chaetomium* sp. and its application in Chinese steamed bread. Food Chem. 120: 457-462.
Knob A, Carmona EC (2010). Purification and Characterization of Two Extracellular Xylanases from *Penicillium sclerotiorum*: A Novel Acidophilic Xylanase. Appl. Biochem. Biotechnol. 162(2): 429-433.
Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680-685.
Li L, Tian H, Cheng Y, Jiang Z, Yang S (2006). Purification and characterization of a thermostable cellulase-free xylanase from the newly isolated *Paecilomyces thermophila*. Enzyme Microb. Technol. 38: 780-787.
Lineweaver H, Burk D (1934). The Determination of Enzyme Dissociation Constants. J. Am. Chem. Soc. 56(3): 658-666.
Lowry H, Rosebrough NJ, Farr AL, Randal RJ (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193(1): 267-275.
Lu F, Lu M, Lu Z, Bie X, Zhao H, Wang Y (2008). Purification and characterization of xylanase from *Aspergillus ficuum* AF-98. Bioresour. Technol. 99: 5938-5941.
McIlvaine TC (1921). A buffer solution for colorimetric comparison. J. Biotechnol. 49: 183-186.
Michelin M, Peixoto-Nogueira SC, Betini JH, da Silva TM, Jorge JA, Terenzi HF, Polizeli MLTM (2010). Production and properties of xylanases from *Aspergillus terricola* Marchal and *Aspergillus ochraceus* and their use in cellulose pulp bleaching. Bioprocess Biosyst. Eng. 33(7): 813-821.
Miller GL (1959). Use of dinitrosalicilic acid reagent for determination of reducing sugar. Anal. Chem. 31: 426-429.
Pal A, Khanum F (2010). Production and extraction optimization of xylanase from *Aspergillus niger* DFR-5 through solid-state-fermentation. Bioresour. Technol. 101(19): 7563-7569.
Peixoto-Nogueira SC, Michelin M, Betini JH, Jorge JA, Terenzi HF, Polizeli MLTM (2009). Production of xylanase by *Aspergilli* using alternative carbon sources: application of the crude extract on cellulose pulp biobleaching. J. Ind. Microbiol. Biot. 36(1): 149-155.
Peixoto-Nogueira SC, Sandrim VC, Guimarães LHS, Jorge JA, Terenzi HF, Polizeli MLTM (2008). Evidence of thermostable amylolytic activity from *Rhizopus microsporus* var. rhizopodiformis using wheat bran and corncob as alternative carbon source. Bioprocess Biosyst. Eng. 31: 329-334.
Polizeli MLTM, Rizzatti AC, Monti R, Terenzi HF, Jorge JA, Amorim DS (2005). Xylanases from fungi: properties and industrial applications. Appl. Microb. Biot. 67(5): 577-591.
Rizzatti ACS, Jorge JA, Terenzi HF, Rechia CGV, Polizeli MLTM (2001). Purification and properties of a thermostable extracellular β-xylosidase produced by thermotolerant *Aspergillus phoenicis*. J. Ind. Microbiol. Biot. 26(3): 156-160.
Ryan SE, Nolan K, Thompsom R, Gubitiz-Savage AV, Tuohy MG (2003). Purification and characterization of a new low molecular weight endoxylanase from *Penicillium capsulatum*. Enzyme Microbiol. Technol. 33(6): 775-785.
Salles BC, Cunha RB, Fontes W, Sousa MV, Filho EX (2000). Purification and characterization of a new xylanase from *Acrophialophora nainiana*. J. Biotechnol. 81(2-3): 199-204.
Sandrim VC, Rizzatti ACS, Terenzi HF, Jorge JA, Milagres AMF, Polizeli MLTM (2005). Purification and biochemical characterization of two xylanases produced by *Aspergillus caespitosus* and their potential for kraft pulp bleaching. Process. Biochem. 40: 1823-1828.
Sapre MP, Jha H, Patil MB (2005). Purification and characterization of a thermostable-cellulase free xylanase from *Syncephalastrum racemosum* Cohn. J. Gen. Appl. Microbiol. 51(6): 327-334.
Savitha S, Sadhasivam ES, Swaminathan EK (2007). Application of *Aspergillus fumigatus* Xylanase for Quality Improvement of Waste Paper Pulp. B. Environ. Contamin. Toxicol. 78(3-4): 217-221.
Singh S, Madlala AM, Prior BA (2003). *Thermomyces lanuginosus*: properties of strains and their hemicellulases. FEMS Microbiol. Rev. 27: 3-16.
Squina FM, Mort AJ, Decker SR, Prade RA (2009). Xylan decomposition by *Aspergillus clavatus* endo-xylanase. Protein Expres. Purif. 68(1): 65-71.
Wong KKY, Tan LUL, Saddler JN (1988). Multiplicity of β-1,4-xylanase in Microrganisms: Funct. Appl. Microbial. Rev. 52(3): 305-317.
Yan Q, Hao S, Jiang Z, Zhai Q, Chen W (2009). Properties of a xylanase from *Streptomyces matensis* being suitable for xylooligosaccharides production. J. Mol. Catal B-Enzyme, 58: 72-77.
Yang SQ, Yan QJ, Jiang ZQ, Li LT, Tian HM, Wang YZ (2006). High-level of xylanase production by the thermophilic *Paecilomyces thermophila* J18 on wheat straw in solid-state fermentation. Bioresour. Technol. 97(15): 1794-1800.
Yeasmin S, Kim CH, Park HJ, Sheikh MI, Lee JY, Kim JW, Back KK, Kim SH (2011). Cell surface display of cellulase activity-free xylanase enzyme on *Saccharomyces cerevisiae* EBY100. Appl. Biochem. Biotechnol. 164(3): 294-304.