J Ind Microbiol Biotechnol (2010) 37:1041–1051
DOI 10.1007/s10295-010-0751-4

ORIGINAL PAPER

Purification and characterization studies of a thermostable β-xylanase from *Aspergillus awamori*

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Received: 18 October 2009 / Accepted: 20 May 2010 / Published online: 15 June 2010

Abstract This study presents data on the production, purification, and properties of a thermostable β-xylanase produced by an *Aspergillus awamori* 2B.361 U2/1 submerged culture using wheat bran as carbon source. Fractionation of the culture filtrate by membrane ultrafiltration followed by Sephacryl S-200 and Q-Sepharose chromatography allowed for the isolation of a homogeneous xylanase (PXII-1), which was 32.87 kDa according to MS analysis. The enzyme-specific activity towards soluble oat spelt xylan, which was found to be 490 IU/mg under optimum reaction conditions (50°C and pH 5.0–5.5), was 17-fold higher than that measured in the culture supernatant. Xylan reaction products were identified as xylobiose, xylotriose, and xylotetraose. K_m values (mg ml^{-1}) for soluble oat spelt and birchwood xylan were 11.8 and 9.45, respectively. Although PXII-1 showed 85% activity retention upon incubation at 50°C and pH 5.0 for 20 days, incubation at pH 7.0 resulted in 50% activity loss within 3 days. PXII-1 stability at pH 7.0 was improved in the presence of 20 mM cysteine, which allowed for 85% activity retention for 25 days. This study on the production in high yields of a remarkably thermostable xylanase is of significance due to the central role that this class of biocatalyst shares, along with cellulases, for the much needed enzymatic hydrolysis of biomass. Furthermore, stable xylanases are important for the manufacture of paper, animal feed, and xylooligosaccharides.

Keywords *Aspergillus awamori* · β-xylanase · Thermostable xylanase · L-cysteine xylanase stabilization

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Introduction

The increasing trend towards the use of renewable, cheap, and readily available biomass for the production of a range of fine and bulk chemicals calls for the development of customized enzyme blends to process biomass in a clean, efficient, and economic manner. Until recently, attention has been predominantly paid to the purification, characterization, and stability of cellulases, including both endoglucanases and exoglucanases. However, the study of enzymes belonging to the xylanolytic system is equally necessary. This study is particularly relevant when the pretreated biomass material retains hemicelluloses. Indeed, this polysaccharide, like cellulose, is, for the most part, unaffected by the use of biomass alkaline or milling pretreatments [33]. Moreover, even in the extensively studied acidic biomass pretreatments, such as steam explosion, which is able to extract and partially hydrolyze most of the biomass hemicelluloses [30], residual amounts of this polysaccharide

remain present in the pretreated material and can hinder cellulose hydrolysis.

Hemicelluloses are the second most abundant renewable polysaccharide and account for 25–35% of lignocellulosic biomass. Hemicelluloses are heterogeneous polymers composed of pentoses (D-xylose, D-arabinose), hexoses (D-mannose, D-glucose, D-galactose), and sugar acids. Depending on the predominant sugar type, the hemicelluloses are referred to as xylans, mannans, or galactans. Hardwood contains mainly xylans, while glucomannans are most common in softwood. The C5 and C6 sugars, which are linked through 1.3, 1.4, and 1.6 glycosidic bonds and often acetylated, form a loose, very hydrophilic structure that promotes an association between cellulose and lignin [1]. Xylan is a branched polysaccharide that is composed of a backbone containing β-1,4-linked-D-xylosyl residues and different side chains, depending on its origin [26]. There are various enzymes responsible for the degradation of hemicellulose. Endo-1,4-β-xylanase (EC 3.2.1.8), β-xylosidase (EC 3.2.1.37), α-glucuronidase (EC 3.2.1.139), α-L-arabinofuranosidase (EC 3.2.1.55), and acetylxylan esterase (EC 3.1.1.72) act in xylan degradation, whereas β-mannanase (EC 3.2.1.78) and β-mannosidase (EC 3.2.1.25) cleave the glucomannan polymer backbone. The main chain endo-cleaving enzymes (xylanases and mannanases) are among the most well-known hemicellulases [53].

Biomass enzyme blends that contain enzymes of the xylanolytic system are able to remove the hemicellulose coating from the cellulose microfibrils [48] and to diminish the lignin barrier to cellulose hydrolysis, as hemicellulose is the linking material between cellulose and lignin [40]; as such, the collective effect of hemicellulases significantly improves the enzymatic hydrolysis of cellulose by cellulases [38]. Furthermore, the use of the xylanolytic system is beneficial in comparison to hydrolysis under acidic conditions, as it results in higher sugar yields and precludes the degradation of pentose sugars into furfural, a metabolic inhibitor. Rich biomass sugar syrups can be used, via chemical or biochemical transformations, to obtain a variety of biorefinery target products in a sustainable manner [52]. Xylanases have also been studied for the production of xylooligosaccharides, which are used as moisturizing agents for food, sweeteners and specific health food, amongst other applications [14, 58, 66]. The activity profile of purified xylanases for xylan hydrolysis, aiming the production of xylooligosaccharides, has also been studied [14]. Xylanases are also important in the pulp and paper industry, particularly in the biobleaching process [49, 54].

This study focused on the purification and characterization of a thermostable β-xylanase excreted by *Aspergillus awamori* that is able to produce high enzyme levels in comparison to reported data [18].

Materials and methods

Chemicals

4-*O*-methyl-D-glucurono-D-xylan, oat spelt xylan, birchwood xylan, *p*-nitrophenyl-β-D-glucuronide (pNPG), carboxymethyl cellulose (CMC), pectin, galactomannan from locust bean gum, *N*-bromosuccinimide (NBS), sodium dodecyl sulfate (SDS), iodoacetamide, *N*-ethylmaleimide (NEM), diethyl pyrocarbonate (DEPC), 1-ethyl-3-(3-dimethylamino-propyl)-carbodiimide (EDC), 2,2-dithiodipyridine, 1,4-dithiothreitol (DTT), β-mercaptoethanol, L-cysteine and L-tryptophan were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Microcrystalline cellulose (Avicel) was purchased from Fluka (Munich, Germany). Filter paper (FP) (Whatman No. 1), Sephacryl S-200 and Q-Sepharose were purchased from GE Healthcare Life Sciences (São Paulo, SP, Brazil). All other chemicals were analytical-grade reagents.

Strain propagation and maintenance

Aspergillus awamori 2B.361 U2/1 strain was used in this study. The Commonwealth Mycological Institute classified this strain in the *Aspergillus niger* complex because it is a sequential mutant of NRRL 3312, which is a member of the *A. niger* series [5]. The *A. awamori* strain has been deposited in the fungi culture collection of the National Institute of Quality Control in Health (INCQS 40259) of the Oswaldo Cruz Foundation (http://www.incqs.fiocruz.br). The fungus was propagated on potato dextrose agar (PDA) plates at 30°C for 7 days, until a dense black sporulation was observed. Spores were collected by adding 2 ml of sterilized distilled water to the plate, followed by a gentle scraping. A sample of the spore suspension was diluted and the spores counted using a Neubauer-counting chamber. A standardized spore suspension presenting 10^6 spores/ml in 20% (v/v) glycerol was maintained at −20°C [4].

Xylanase production

The volume of 3 ml of the spore suspension presenting 10^6 spores/ml was inoculated in a growth medium containing (%w/v): 0.6 yeast extract, 3.0 wheat bran, 0.12 $NaNO_3$, 0.3 KH_2PO_4, 0.6 K_2HPO_4, 0.02 $MgSO_4 \cdot 7H_2O$ and 0.005 $CaCl_2 \cdot 2H_2O$, initial pH 7.0. One-liter Erlenmeyer flasks containing 300 ml of the medium were incubated at 200 rpm and 30°C for up to 7 days. Xylanase activity was measured throughout the incubation period and cultures bearing peak activity were filtered using a glass fiber filter in a vacuum pump system and the

filtrate was used for xylanase purification and biochemical studies.

Enzyme production experiments were carried out in triplicates and xylanase activity values were reported as average with an indicated standard deviation.

Enzyme purification using membrane ultrafiltration, gel filtration, and ion-exchange chromatography

The filtered culture supernatant was ultrafiltrated using a 300-kDa membrane (Amicon Filtration System-Stirred Cells) for the removal of high-molecular-weight proteins. The retentate was discharged, and the ultrafiltrate, which presented 87% of the culture filtrate enzyme activity, was subsequently fractionated using a 100-kDa membrane.

A volume of 10 ml of the PM100 retentate, which contained 22.05 mg of protein and a total xylanase activity of 447.3 IU, was subsequently fractionated by gel filtration on a Sephacryl TM S-200 High-Resolution Column (3.0 × 42 cm) that was pre-equilibrated with 50 mM sodium acetate buffer (pH 5.0) containing 0.15 M NaCl. The sample was eluted using a flow rate of 20 ml/h, and 5.0 ml aliquots were collected and screened for xylanase activity and protein concentration through absorbance at 280 nm. Fractions presenting xylanase activity were pooled and dialyzed against 50 mM sodium phosphate buffer (pH 7.0). Subsequently, 15-ml aliquots, which contained 1.98 mg of protein and a total xylanase activity of 405.6 IU, were analyzed by ion-exchange chromatography using a Q-Sepharose column (2.5 × 8.4 cm). The column was pre-equilibrated and eluted with the same sodium acetate buffer followed by a linear NaCl gradient (0–1 M). The sample was eluted using a flow rate of 30 ml/h, and the 5.0-ml aliquots presenting xylanase activity were collected and pooled. Protein concentration was also measured according to Bradford [7].

Xylanase activity, kinetic parameters, and substrate specificity

The measurement of the enzyme activity was performed at pH 5.5 and 50°C, according to Filho et al. [25]. One unit (IU) of xylanase activity corresponded to the release of 1 µmol of reducing sugar per minute. The concentration of reducing sugars was measured according to Miller et al. [46] using xylose as standard.

Kinetic parameters for *A. awamori* purified xylanase were determined for the soluble and insoluble fractions of oat spelt and birchwood xylan, which were prepared according to Filho et al. [25]. Untreated birchwood xylan, prepared as described by Bailey et al. [2], was also used in the kinetic assays. Kinetic experiments were performed using substrates in a concentration range of 2.67–26.7 mg/ml. K_m and V_m values were estimated using the Michaelis–Menten equation in a non-linear regression data-analysis program [41]. The purified xylanase preparation was also tested against 1% (w/v) 4-*O*-methyl-D-glucurono-D-xylan, pNPG, CMC, pectin, and 0.5% (w/v) galactomannan in routine assay conditions. Filter paper (FP) and microcrystalline cellulose (Avicel) were also tested [64].

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Xylanase preparations from gel filtration and ion-exchange chromatography were analyzed by SDS-PAGE using a 15% polyacrylamide gel [39] containing 0.1% (w/v) oat spelt xylan. Upon the completion of electrophoresis, the gel was divided and analyzed for protein bands using the Coomassie Blue G-250 dye [10] or xylanase activity. For zymogram analysis, the gel was treated with Triton X-100 (1%) for 30 min at 4°C and incubated for 10 min at 50°C in 50 mM citrate-phosphate buffer (pH 5.0) to foster xylanase activity. The gel was subsequently incubated under agitation at room temperature in 0.1% (w/v) Congo Red for 10 min and washed with 1 M NaCl for the visualization of clear bands, which indicated xylanase activity [47]. Low-molecular-weight standards from Sigma were used as molecular mass markers.

Mass spectrometry

A selectively pooled sample from the Q-Sepharose chromatography was lyophilized and solubilized in 100 µl trifluoroacetic acid (TFA) (0.1%). A 1-µl sample mixed with 1 µl sinapinic acid (20 µg/µl) was placed on a mass spectrometer stainless-steel plate. The MS analysis was performed under the linear positive mode on a Bruker Daltonics Autoflex II MALDI-TOF/TOF mass spectrometer. The mass range was 4–70 kDa, and external calibration was performed with cytochrome C. Data was collected and analyzed with Bruker Daltonics FlexControl 2.4 and FlexAnalysis 2.4 software, respectively.

Effect of pH and temperature on xylanase activity

Activity of the culture filtrate and purified xylanase was measured at pH 3.0–6.0 (50 mM sodium acetate buffer), 6.0–7.5 (50 mM sodium phosphate buffer), and 7.5–9.0 (50 mM Tris–HCl buffer) at 50°C. The ionic strength of the buffer was adjusted with NaCl when necessary. For the evaluation of the effect of temperature, xylanase activity

was measured at the temperature range of 30–80°C at pH 5.0. Experiments were done in triplicate and average values were reported as normalized activity. Standard deviations were less than 10%.

Identification of the hydrolysis products of oat-spelt xylan

Purified xylanase (10.37 IU/ml) was used in the soluble oat-spelt xylan hydrolysis experiments, which were conducted at pH 5.5 and 50°C. Reactions, which were incubated for 2, 4, 8, or 16 min, were quenched by boiling followed by the measurement of the reducing sugars [46]. The fractionation of the oligosaccharides pool was carried out by thin-layer chromatography (TLC) [66]. Samples of the reaction mixtures, presenting 20 μg of xylose-equivalent reducing sugars, were applied to a chromatography sheet that was drawn up by a solvent mixture of methanol: *n*-butanol:H_2O in the proportion of 5:5:3. A xylooligosaccharides standard mixture (20 μg/μl) was also used. The sugar spots were identified by spraying 0.2% orcinol dissolved in 20% sulfuric acid, followed by heating [66].

Xylanase thermostability

Purified xylanase was incubated at 28°C (pH 5.0 and 5.5), 50°C (pH 5.0, 5.5 and 7.0), and 55°C (pH 7.0). Depending on the pH, the enzyme samples were previously dialyzed against 50 mM sodium acetate buffer for pH 5.0 and 5.5, or 50 mM sodium phosphate buffer for pH 7.0. Thermostability experiments, at 50 and 55°C, pH 7.0, were also performed in the presence of either 20 mM L-cysteine, L-tryptophan or 10 mM DTT. Residual activity was measured in samples taken throughout the experiment under standard conditions. Xylanase thermostability experiments were done in duplicate and average values were reported.

Effect of modifying reagents, amino acids, chloride ions, and sulfate ions on xylanase activity

Purified xylanase activity was investigated in the presence of 10 mM NBS, SDS, iodoacetamide, NEM, DEPC, EDC, 2,2-dithiodipyridine and DTT as well as in the presence of 20 mM *β*-mercaptoethanol, L-cysteine and L-tryptophan. The effect of 10 mM chloride (KCl, $CaCl_2$, $ZnCl_2$, $MnCl_2$, NaCl, CoCl, $MgCl_2$, $HgCl_2$), sulfate salts ($CuSO_4$ and $FeSO_4$) and ethylenediamine tetra acetic acid (EDTA) was also investigated after enzyme preincubation for 20 min at 28°C in the presence of the relevant salt. Appropriate controls were included in all cases [24]. Experiments were done in triplicate and average values were reported as normalized activity. Standard deviations were less than 10%.

Results and discussion

Aspergillus awamori xylanase production

According to data presented in Table 1, xylanase accumulation peaked within 4 days of incubation, reaching 19.0 IU/ml. This value correlates with the literature, as Poutanen et al. [50] reported a xylanase concentration of 12 IU/ml for *A. awamori* VTT-D-75028 cultivated in wheat bran. Equivalent enzyme levels (22.2 IU/ml) were also reported for *A. carneus* M34 in submerged fermentation using oat-spelt xylan as carbon source [22]. Lower enzyme accumulation (9.75 IU/ml) was observed for *Aspergillus nidulans* CECT 2544 and *Aspergillus* sp. PK-7 (10.6 IU/ml) [32]. The cultivation of the *A. awamori* strain studied in the present work, using the agro-residue grape pomace in solid-state fermentation, yielded 35 IU/g [6].

Ultrafiltration of the filtered culture supernatant and xylanase purification by gel filtration and ion-exchange chromatography

Table 2 summarizes the data for the xylanase purification steps. The ultrafiltration data indicate that 87.14% of the total activity from the culture filtrate was recovered in the PM 300 ultrafiltrate, whose specific activity (35.08 IU/mg) was higher than that of the filtrate (29.02 IU/mg). The subsequent ultrafiltration of the PM 300 ultrafiltrate using a 100-kDa membrane resulted in the recovery of the bulk enzyme activity in the PM 100 ultrafiltrate (3,713.85 IU) as expected, considering that the molecular mass of the xylanases fell in the range of 46–13 kDa (Table 3). However, as the PM100 retentate was also a rich xylanase preparation (447.3 IU), chromatographic purification studies were furthered using this fraction even though its specific activity (20.29 IU/mg) was lower that of the filtrate (41.58 IU/mg). The protein and activity elution profile of the PM 100 retentate from the Sephacryl S-200 step is shown in Fig. 1. This chromatographic step separated the xylanase protein quite well, as 90% of the activity was detected in a low protein concentration elution region (PXI) and was well separated from a subsequently eluted,

Table 1 Time course for xylanase accumulation in the supernatant of *Aspergillus awamori* culture

Time (days)	IU/ml
1	2.11 ± 0.16
2	8.86 ± 0.44
3	12.70 ± 0.52
4	19.37 ± 0.11
5	17.52 ± 3.27
6	4.31 ± 0.10
7	5.86 ± 0.24

Table 2 Data for the separation and purification steps of *A. awamori* xylanase

Purification steps	Total protein (mg)	Total activity (IU)	Specific activity (IU/mg)	Purification (fold)	Xylanase yield (%)
Culture supernatant	184.17	5,345	29.02	1.00	100.00
Ultrafiltration					
Retentate PM 300	58.17	725.5	12.47	0.43	13.57
Ultrafiltrate PM 300	132.75	4,657.5	35.08	1.21	87.14
Retentate PM 100	22.05	447.3	20.29	0.70	8.37
Ultrafiltrate PM 100	89.33	3,713.85	41.58	1.43	69.48
Sephacryl S-200	1.98	405.6	204.85	7.06	7.59
Q-Sepharose	0.5	245	490.00	16.88	4.58

Table 3 Reported physical and chemical properties of xylanases produced by fungi of the genus *Aspergillus*

Species	Enzyme	Molecular mass (kDa)	pH_{opt}	T_{opt} (°C)	*p*I	Reference
A. awamori CMI	I	39	5.5–6.0	55	5.7–6.7	[37]
A. awamori CMI	II	23	5.0	50	3.7	[37]
A. awamori CMI	III	26	4.0	45–50	3.3–3.5	[37]
A. niger	XYLI	20.8	5.0	55	6.7	[28]
A. niger	XYLII	13	6.0	45	8.6	[29]
A. niger	XYLIII	13	5.5	45	9.0	[29]
A. niger	XYLIV	14	4.9	45	4.5	[56]
A. niger	XYLV	28	5.0	42	3.65	[27]
A. caespitosus	Xyl I	27	6.5–7.0	50–55		[54]
A. caespitosus	Xyl II	17.7	5.5–6.5	50–55		[54]
A. fischeri		31	6.0	60		[51]
A. ficuum AF-98		35	5.0	45		[23]
A. fumigatus	II	19	5.5	55		[57]
A. kawachii	XylA	35	5.5		6.7	[34]
A. oryzae		46.5	5.0	55	3.6	[31]
A. carneus M34		18.8	6.0	50	7.7–7.9	[21]
A. versicolor	II	32	6.0–7.0	55		[13]
A. awamori 2B.361 U2/1	PXII-1	32.87	5.0–5.5	50		Present work

lower molecular mass bulk protein peak. The specific activity of the PXI preparation was ten-fold higher (204.85 IU/mg) in comparison to that of the PM100 retentate (20.29 IU/mg). The elution and activity profiles of the PXI preparation from the anion exchange Q-Sepharose chromatography, which are shown in Fig. 2, indicate the existence of two major xylanase isoforms that were closely eluted as well as two minor, more acidic isoforms that were eluted after the application of the salt gradient. The results of the present study corroborate with the literature, as Kormelink et al. [37] reported the existence of three xylanase isoforms with isoelectric points ranging from 6.7 to 3.3 for *A. awamori* CMI (Table 3). The specific activity of xylanase PXII-1 was 24-fold higher (490 IU/mg) in comparison to that of the PM100 retentate (20.29 IU/mg). The chromatographic procedures allowed for the recovery of 50% of the total enzyme activity from the PM100 retentate. The protein fractions corresponding to the PXI and PXII-1 xylanase peaks were pooled separately and dialyzed for further studies.

SDS-PAGE and mass spectrometry analysis

According to data presented in Fig. 3a, SDS-PAGE fractionation of the PXI preparation shows nine protein bands with molecular weights ranging from 97 to 14 kDa. SDS-PAGE analysis of PXII-1 xylanase shows that this preparation migrated as a homogeneous single band with a molecular weight of 32 kDa, suggesting a monomeric protein structure. Zymogram (Fig. 3b) of the PXII-1 preparation showed the presence of a hydrolysis zone that was coincident to the single PXII-1 protein band. The

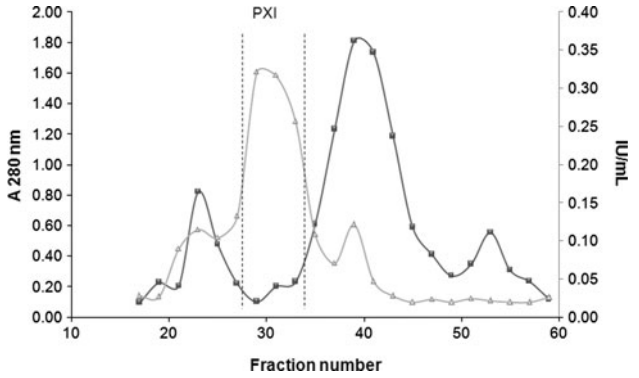

Fig. 1 Sephacryl S-200 chromatography elution profiles of protein (—■—) and xylanase activity (—△—) of the 100-kDa retentate. The culture filtrate was fractionated by ultrafiltration, and the 100-kDa retentate was applied to the column. The xylanase activity peak and the corresponding PXI protein fractions are also shown

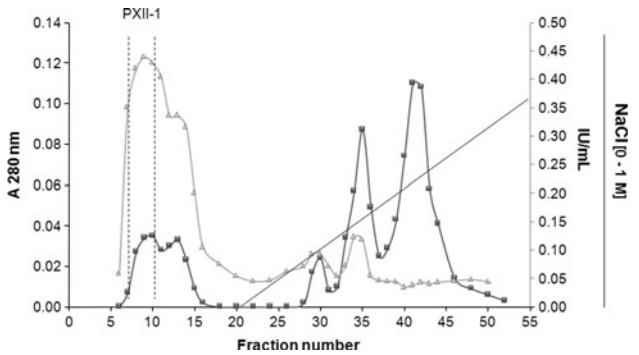

Fig. 2 Q-Sepharose chromatography elution profiles of the pooled PXI protein sample from Sephacryl S-200 chromatography. The protein (—■—) and xylanase activity (—△—) profile is shown as well as the activity peak corresponding to the PXII-1 protein fraction. NaCl gradient (*continuous line*)

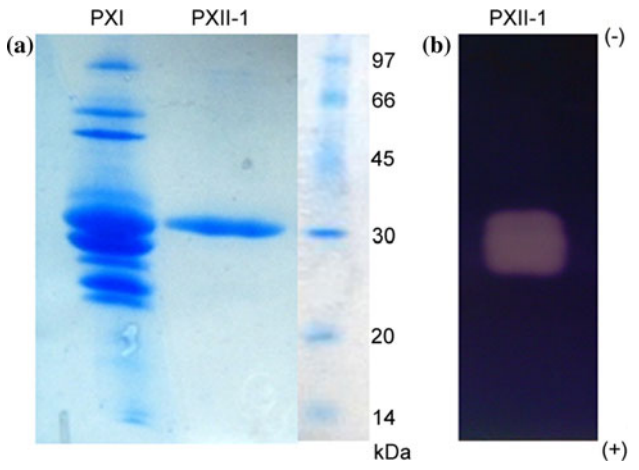

Fig. 3 Protein analysis of the preparation PXI from Sephacryl S-200 chromatography and the preparation PXII-1 from Q-Sepharose chromatography by SDS-PAGE (**a**). The zymogram of the PXII-1 preparation is also shown (**b**)

homogeneity of this preparation was further confirmed by mass spectrometry (MALDI-TOF), which showed a unique protein peak with a molecular mass of 32.87 kDa, a value that compares well to previously reported data on *Aspergillus* xylanases (Table 3). The molecular mass of PXII-1 xylanase was within the range detected for xylanases belonging to the G/11 family [62]. Low molecular mass and thermostable xylanases are of industrial importance because they can better diffuse into the biomass structure or fibrous pulp and can thus efficiently hydrolyze xylan in biomass hydrolysis or pulp bleaching [26, 45].

Effect of pH and temperature on enzyme activity

Optimum xylanase activity for both the culture filtrate and PXII-1 preparation was observed within the pH range 5.0–5.5 and the temperature range of 50–60°C (Fig. 4). Nevertheless, an evident activity decrease was observed above 60°C. These findings are in agreement with those reported by Shah and Madamwar [55] and Coelho and Carmona [16], who reported that the optimum temperature was 50°C and the optimum pH was 5.3 and 6.0, respectively, for *Aspergillus foetidus* and *Aspergillus giganteus* xylanases. According to Table 3, the optimum temperature and pH value of xylanases from *Aspergillus* species ranges from 42 to 55°C and 4.0 to 6.0, respectively.

Within this context, it is worthwhile to mention that some biotechnological applications for xylanase, such as for biomass hydrolysis and as animal feed additive, call for activity in the pH and temperature ranges of 4.8–5.5 and 40–50°C, respectively [9, 59].

Xylanase substrate specificity and kinetic parameters

Kinetic parameters for PXII-1 on soluble and insoluble fractions of oat-spelt and birchwood xylan are presented in Table 4. The overall data for the apparent K_m indicate that *A. awamori* xylanase shows higher affinity for the less branched birchwood xylan, which contains 90% xylose [51], in comparison to oat-spelt xylan, which contains 75% xylose, 10% arabinose, and 15% glucose [19]. However, the K_m value of the PXII-1 preparation was higher in comparison with other *Aspergillus*-purified xylanases, such as that from *A. fischeri* (K_m 4.88 mg/ml) [51], *A. caespitosus* (K_m 3.9 mg/ml) [54] and *A. ficuum* AF-98 (K_m 3.75 mg/ml) on birchwood xylan [23] and *A. versicolor* (K_m 2.3 mg/ml) on oat-spelt xylan [13]. Xylanase PXII-1 also showed the highest K_{cat} and K_{cat}/K_m values towards untreated birchwood xylan. Xylanase PXII-1 was also active on 4-*O*-methyl-D-glucuronoxylan (2.06 IU/ml). A very low activity, less than 0.05 IU/ml, was detected for galactomannan and pectin, whereas no enzyme activity was detected for pNPG, Avicel, filter paper, or CMC. This

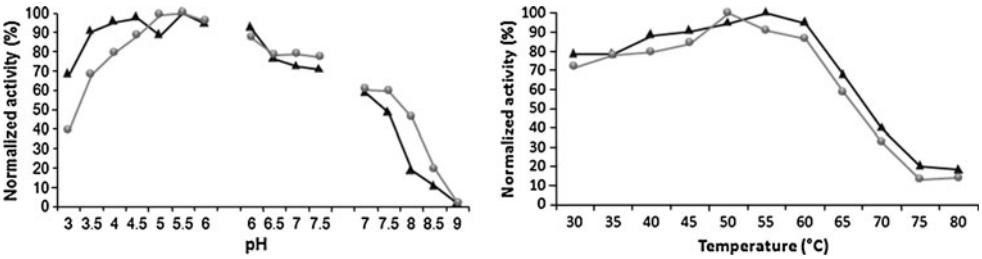

Fig. 4 Effect of pH and temperature on xylanase activity from the culture filtrate (▲) and on the PXII-1 xylanase preparation (●)

Table 4 Kinetic parameters of *A. awamori* PXII-1 xylanase

Kinetic parameter	Oat-spelt xylan		Birchwood xylan		
	Soluble	Insoluble	Untreated	Soluble	Insoluble
K_m (mg/ml)	11.8	15.31	9.75	9.45	10.17
V_{max} (IU)	6.98	3.03	7.98	2.47	3.89
K_{cat} (min^{-1})	1.745	0.758	1.995	0.618	0.973
K_{cat}/K_m (ml/min mg protein)	0.14	0.049	0.205	0.065	0.096

result is consistent with the substrate specificity of purified xylanases from *A. versicolor* [12, 13], *A. caespitosus* [54], and *A. fischeri* [51].

Xylanase hydrolytic profile

The TLC analysis of the products resulting from the hydrolysis of oat-spelt xylan by purified xylanase is presented in Fig. 5. Within the 16-min reaction time, the hydrolysis products evolved towards the predominant formation of xylobiose, xylotriose, and xylotetraose, nevertheless small xylose amounts were also detected. Results suggest that the chain size of the xylooligosaccharides (XOs) pool could be designed upon the use of specific reaction conditions where reaction time would be an important parameter. These results confirm that xylanase PXII-1 is a xylan endo-acting enzyme. Purified endoxylanase from *A. carneus* [21] and *A. niger* [43] showed a similar product profile nevertheless only xylooligosaccharides were detected upon xylan hydrolysis by *A. versicolor* xylanase [12]. Enzymes with high endoxylanase activity and low exo-xylanase and/or β-xylosidase activity are favored for the production of xylooligosaccharides (XOs) [63] that can be used as prebiotics. The xylanase PXII-1, which is abundantly produced and easily purified, has biotechnological importance for xylooligosaccharide production.

Xylanase thermostability

PXII-1 xylanase retained more than 85% activity after 35 days of incubation at 28°C and after 20 days of incubation at 50°C at either pH 5.0 or 5.5. However, this thermostable xylanase proved to be more temperature sensitive at a higher pH, as the incubation at pH 7.0 at 50 and 55°C

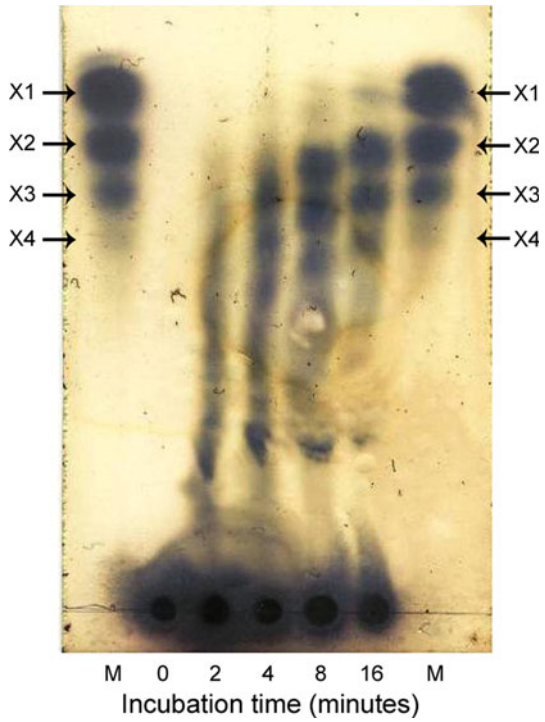

Fig. 5 TLC analysis of products resulting from the hydrolysis of oat-spelt xylan by purified xylanase according to the incubation time of the reaction mixture. Sugar standards (*M*) correspond to xylose (*X1*), xylobiose (*X2*), xylotriose (*X3*), and xylotetraose (*X4*)

resulted in 50% activity loss within 3 days and about 1 h, respectively. The effect of incubation time on thermostability of xylanase PXII-1 at pH 7.0 is shown in Fig. 6. However, PXII-1 xylanase stability at pH 7.0 was greatly improved in the presence of 20 mM L-cysteine, as 85% of activity retention was observed for 25 days at 50°C and for 1 day at 55°C (Fig. 7). The observed protective effect of L-cysteine could be related to the presence of a reducing

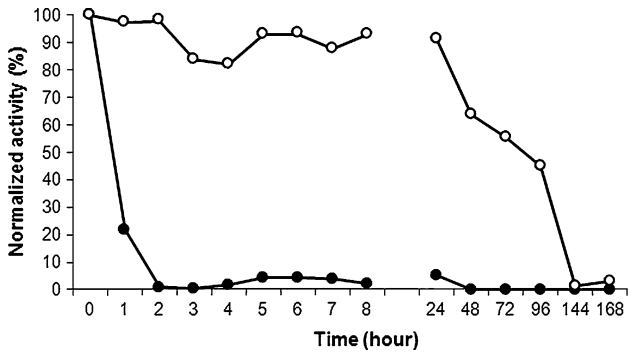

Fig. 6 Effect of incubation time on the thermostability of the PXII-1 xylanase preparation at pH 7.0, and 50°C (—○—) or 55°C (—●—)

environment that would keep L-cysteine residue monothiols in a reduced state, avoiding the formation of—S–S—bridges that could impair the native tertiary structure of the protein. As such, L-cysteine residues could play a key role on either the overall stability of xylanase and/or the structure of the enzyme active site. Incubation in the presence of L-tryptophan and 10 mM DTT did not alter the overall stability data presented in Fig. 6 (data not shown).

The half-lives for several fungi xylanases at 50°C have been reported as 1 h for *Aspergillus foetidus* [55], 13 min for *A. giganteus* [16], and 4 h for *A. awamori* NRRL 3112 [42]. Concerning stability at 55°C, xylanase II purified from *A. caespitosus* was fully stable for up to 90 min [54]. Although the remarkably lower stability of the aforementioned preparations, as compared to the *A. awamori* xylanase PXII-1 studied here, might be due to an unfavorable pH environment, it is beyond doubt that the enzyme evaluated in the present study is particularly stable. This enzyme would be suitable for biomass hydrolysis experiments, which are typically carried out at pH 5.0 and 45–50°C [15, 61] to allow for easier mixing, better substrate solubility, high mass transfer rate, and lower risk of contamination. It has also been reported that the addition of xylanase to a pretreated biomass at 45°C for 72 h increased the amount of glucose recovered, reaching almost 100% of the total theoretical glucose [48]. Also considering the aforementioned hydrolytic profile of the products that were obtained from oat-spelt xylan hydrolysis, *A. awamori* xylanase PXII-1 would be adequate for the production of xylooligosaccharides.

Effect of modifying reagents, amino acids, chloride ions, and sulfate ions on xylanase activity

The inhibition or activation of xylanases by selected chemicals is useful for the study of the structure of the active site and its mechanism of action. The effects of amino acid-modifying agents, amino acids, chloride ions, sulfate ions, and EDTA on PXII-1 xylanase activity are shown in Tables 5 and 6. The presence of L-tryptophan in the reaction mixture increased xylanase activity by 37%, and a similar increase was observed for cysteine, confirming the presence of reduced thiol (cysteine) in the enzyme structure [13]. Similar results were found in the characterization of xylanase from *Acrophialophora nainiana* [65], which was demonstrated to be activated by cysteine and tryptophan; *Clostridium thermocellum* [64], which is activated by cysteine, tryptophan, and DTT; *Penicillium capsulatum* [25], which is activated by cysteine and DTT; *Aspergillus niger*, *Penicillium corylophilum* and *Trichoderma longibrachiatum* [11], which are activated by 9.3 mM of cysteine and tryptophan; and *Trichoderma harzianum* [24], which is activated by 20 mM of cysteine and DTT. Tests with xylanase from *Streptomyces*, *Bacillus*, and *Chainia* [20, 35] revealed the involvement of tryptophan and cysteine residues in the active sites. It was also demonstrated that the addition of xylan with NBS protects the *Bacillus stearothermophilus* xylanase from inactivation, indicating that the tryptophan residue was present in the active site of the enzyme [36].

The enzyme was fully inactivated in the presence of NBS, which is primarily involved in oxidation of tryptophan residues, although it can also oxidize tyrosine, histidine, and methionine residues. As such, tryptophan might be involved in the active site, participating in binding and/or hydrolysis of the substrate [36]. A mild inhibition of enzyme activity in the presence of alkylating reagents (NEM and iodoacetamide) was also observed, suggesting that the enzyme requires thiol groups for the stability of its structure.

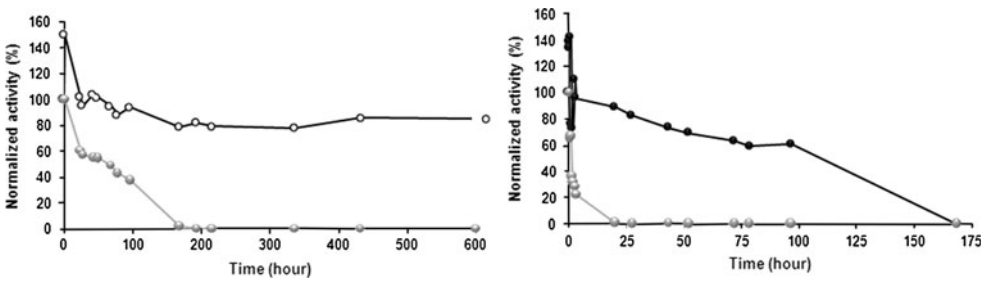

Fig. 7 Effect of 20 Mm L-cysteine on the thermostability of the PXII-1 xylanase preparation at pH 7.0, and 50°C (—○—) or 55°C (—●—), and controls (—●—)

Table 5 Effect of modifying reagents and amino acids on the activity of *A. awamori* PXII-1 xylanase

Reagent	Concentration (mM)	Normalized activity (%)
NBS	10	0.00
SDS	10	57.75
Iodoacetamide	10	78.38
NEM	10	80.87
DEPC	10	81.60
EDC	10	82.50
β-Mercaptoethanol	20	100.03
2,2-Dithiodipyridine	10	104.40
L-Cysteine	20	127.66
DTT	10	135.99
L-Tryptophan	20	137.11
Control[a]	–	100.00

[a] Activity of the purified PXII-1 xylanase −2.83 IU/ml

Table 6 Effect of metal ions and EDTA on the activity of *A. awamori* PXII-1 xylanase

Reagent (10 mM)	Normalized activity (%)
$HgCl_2$	0.00
$CuSO_4$	84.13
EDTA	92.25
$ZnCl_2$	95.23
$FeSO_4$	98.70
$CaCl_2$	100.59
NaCl	100.59
$MgCl_2$	102.46
KCl	104.28
CoCl	113.09
$MnCl_2$	134.99
Control[a]	100.00

[a] Activity of the purified PXII-1 xylanase −2.83 IU/ml

In the presence of DEPC and EDC, the PXII-1 xylanase activity decreased 18.40 and 17.50%, respectively. The failure of DEPC and EDC to stimulate xylanase activity rules out the contribution of histidine and carboxyl groups in binding or catalysis [11, 25]. The involvement of carboxylic groups in the mechanism of xylanase catalysis was reported in studies with *Schizophyllum commune* [8] and *Streptomyces* sp. [44].

The reagent 2,2-ditiodipiridina, which is a sulfhydryl oxidizing agent, was innocuous for the xylanolytic activity; it might be possible that the enzyme thiol groups were inaccessible to this reagent [17] or the reaction conditions, including the reagent concentration were not adequate. The reagent *β*-mercaptoethanol, which cleaves disulfide bonds, did not cause any change in xylanase activity.

The effects of ions and EDTA on xylanolytic activity are presented in Table 6. Hg^{2+} inhibited 100% of xylanase activity, which was likely due to its interaction with sulfydryl groups, suggesting the presence of a cysteine residue as part of the enzyme active site or nearby [3]. This result is in accordance with the literature that reports Hg^{2+} as a potent inhibitor (70 to 100% inhibition) of *Aspergillus* xylanase produced by *A. fischeri* Fxn 1 [51], *A. versicolor* [12], *A. caespitosus* [54], and *A. niveus* RS2 [60].

The presence of Cu^{2+}, Zn^{2+}, Fe^{2+}, Ca^{2+}, Na^{+}, Mg^{2+}, K^{+} and Co^{2+} ions and EDTA did not affect the xylanase activity. However, a 35% increase in activity in the presence of Mn^{2+} was observed. The positive effect of Mn^{2+} was confirmed by incubating xylanase for 30 min in the presence of Mn^{2+}, whereby a 64% increase in activity was observed. Carmona et al. [13] reported that the presence of 10 mM Mn^{2+} stimulated the activity of purified xylanase from *Aspergillus versicolor* in more than 100% of activity. The xylanase purified from *A. niveus* RS2 was also slightly stimulated in the presence of Mn^{2+} [60].

Conclusions

A novel, low-molecular-weight (32.87 kDa), highly thermostable *β*-xylanase produced by *Aspergillus awamori* 2B.361 U2/1 was purified and characterized. The fungus excreted *β*-xylanase in high yields (19,000 IU/l) in submerged cultivation, using a growth medium with wheat bran as a carbon source. The affinity of PXII-1 xylanase (K_m value of 9.45 mg/ml) for soluble birchwood xylan was higher than that measured for soluble oat spelts (11.8 mg/ml). The enzyme showed outstanding thermostability in comparison to that previously reported for *Aspergillus* xylanases, as it retained 85% activity after 20 days of incubation at 50°C at either pH 5.0 or 5.5. The enzyme was more temperature-sensitive at pH 7.0, as incubation at 50 or 55°C resulted in 50% activity loss within 3 days and 1 h, respectively. Nevertheless, enzyme stability at pH 7.0 was greatly increased in the presence of 20 mM L-cysteine. Mn^{2+} activated xylanase activity, while Hg^{2+} inhibited 100% of activity, which was likely due to its interaction with the enzyme cysteine residues. The PXII-1 endoxylanase activity profile was confirmed by the analysis of the oat-spelt xylan hydrolysis products, which were identified as xylobiose, xylotriose, and xylotetraose. The remarkable thermostability of *Aspergillus awamori* 2B.361 U2/1 xylanase is advantageous for biomass hydrolysis and for the production of xylooligosaccharides.

Acknowledgments This work was supported by research and scholarship grants from the National Council for Scientific and Technological Development (CNPq) of the Brazilian Ministry of Science and Technology and from a scholarship grant from the Brazilian Federal Agency for Support and Evaluation of Graduate Education (CAPES) of the Ministry of Education. We are thankful to Dr. Marília Martins Nishikawa from the National Institute of Quality Control in Health (INCQS) of the Oswaldo Cruz Foundation for preserving the *Aspergillus awamori* strain.

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