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Characterization of multiple xylanase forms from *Aspergillus tamarii* resistant to phenolic compounds

Monclaro AV[1], Aquino EN[1,2], Faria RF[1], Ricart CAO[2], Freitas SM[3], Midorikawa GEO[4], Miller RNG[4], Michelin M[5], Polizeli MLTM[5] and Filho EXF[1]

[1]Enzymology Laboratory, University of Brasília, Campus Universitário Darcy Ribeiro, Brasília - DF, 70910-900. e-mail: eximenes@unb.br
[2]Biochemistry and Protein Chemistry Laboratory, University of Brasília, Campus Universitário Darcy Ribeiro, Brasília - DF, 70910-900
[3]Molecular Biophysics Laboratory, University of Brasília, Campus Universitário Darcy Ribeiro, Brasília - DF, 70910-900
[4]Microbiology Laboratory, University of Brasília, Campus Universitário Darcy Ribeiro, Brasília - DF, 70910-900
[5]Microbiology and Cellular Biology Laboratory, University of São Paulo, Ribeirão Preto – SP, 14040-901

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Abstract

Aspergillus tamarii was cultivated in different textile wastes. Xylanases with high levels of enzymatic activity were produced after two days cultivation, with constant production for up to seven days. Two xylanases, namely Xyl-1 and Xyl-2, with molecular masses of 35.5 and 22 kDa, respectively, were isolated from the crude extract and purified by ultrafiltration and gel filtration chromatography. Xyl-1 and Xyl-2 were more active at pH 6.0, and 60° C and 40° C, respectively. The respective K_M and V_{max} values on soluble oat spelt xylan were 4.30 $mg.mL^{-1}$ and 0.249 $IU.mL^{-1}$ (Xyl-1) and 18.92 $mg.mL^{-1}$ and 1.103 $IU.mL^{-1}.s^{-1}$ (Xyl-2). Dynamic light scattering (DLS) was used to evaluate purification steps, effective in assessing the degree of purity of the samples, the presence of aggregations and the size of the enzymes. Tween-80 at 0.1% was an efficient dispersing agent for avoiding aggregation of proteins and did not influence enzyme activity. Purified and partially purified xylanases were activated with auto-hydrolysis liquor from corncob and with ferulic acid, a phenolic compound derived from lignocellulosic biomass. These findings of this study indicate that *A. tamarii* produces multiple forms of xylanases with considerable potential in different biotechnological applications.

Keywords – Corncob autohydrolysis liquor – dynamic light scattering – ferulic acid – textile wastes

Introduction

The plant cell wall of lignocellulosic biomass, which is an important component of agro-industrial residues, is a complex structure composed of three principal components: cellulose, hemicellulose and lignin (Siqueira et al. 2009). Within the context of biotechnological

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Corresponding Author: Filho EXF – e-mail – eximenes.1@gmail.com

applications, second generation bioethanol conversion from agro-industrial wastes represents a promising renewable energy source and an alternative to the use of fossil resources. The polysaccharides within plant cell walls are arranged in an organized manner, forming structures that are highly recalcitrant to enzymatic degradation (Siqueira et al. 2009). As such, efficient hydrolysis of plant cell wall material is one of the main challenges for the biofuel industry.

Xylan is the most abundant component of hemicellulose. It is a heteropolysaccharide with a varied structure, which essentially consists of β-1,4-xylose units in the backbone and L-arabinofuranosyl and methyl glucuronic acid as side groups. The xylose units can be acetylated and L-arabinofuranosyl may have additional side groups such as ferulic acid and ρ-coumaric acid. These residues can be cross-linked with lignin, thus maintaining the integrity of plant cell walls (Polizeli et al. 2005).

Brazil is today one of the major global cotton producers, with cultivation of this cash crop contributing significantly to the national economy (Siqueira et al. 2009). One of the environmental problems related to the cultivation of cotton is the amount of cotton residue generated, which can be five times higher than the total amount of fiber produced (Agblevor et al. 2006). The conversion of this residue into valuable by-products is currently under extensive study, with potential application identified in the biorefinary industry for fuel production (Caldeira-Pires et al. 2013).

Most industrial enzymes are produced by bacteria, yeasts and fungi that are able to ferment specific substrates. A number of fungi from the genus *Aspergillus* are effective decomposers of lignocellulosic biomass and efficient producers of xylanases (Moreira et al. 2013). *Aspergillus tamarii* and *Aspergillus flavus* are closely related species, with *A. tamarii* a non-aflatoxin producer (Ito et al. 2001) and known to be a xylanase producer (Ferreira et al. 1999, Souza et al. 2001).

This study describes the characterization of xylanases in *A. tamarii* BLU37, a strain isolated from natural composting of textile residues in Brazil with potential for application in the biorefinary and biofuel industries. We evaluated both the fungal strain's ability to produce xylanases using different textile residues as a carbon source, as well as the influence of different phenolic compounds on enzyme activities.

Materials & Methods

Residue and chemicals

All reagents and substrates were purchased from Sigma Chemical Co. (St. Louis, USA). Sephacryl S-100, Sephadex G-50 and HiTrap Q FF were purchased from GE Healthcare (São Paulo, Brazil). Cotton residues were donated by Hantex Resíduos Têxteis Ltda (Gaspar, Brazil). All experiments were carried out in quintuplicate. The standard deviation was less than 20% of the mean.

Residue pretreatment

Filter powder (FP) and clean cotton residue (CC) were pretreated as previously described by Duarte et al. (2012). The pretreated filter powder and pretreated clean cotton residue are hereafter referred to as tFP and tCC, respectively.

Organism and enzyme production

A. tamarii BLU37 was originally isolated from natural composting of textile industry residues and deposited in the fungal collection at The Enzymology Laboratory, University of Brasília, Brazil (gene pool access authorization number 010237/2015-1). Ribosomal DNA Internal Transcribed Spacer (rDNA ITS) regions, together with β-tubulin and calmodulin genes, were used as conserved molecular markers to identify the fungus to species level (Midorikawa et al. 2008). The isolate was maintained in PDA medium (2% potato broth, 2% dextrose and 2%

agar). An aliquot (5 mL) of spore suspension (10^8 spores.mL^{-1}) was inoculated into Erlenmeyer flasks containing 500 mL of liquid medium adjusted to pH 7.0 and containing 1.0% (w/v) of FP, tFP, CC and tCC as carbon sources. Two liquid media were employed: a standard liquid medium composed of (w/v) 0.7% KH_2PO_4, 0.2% K_2HPO_4, 0.05% $MgSO_4.7H_2O$, 0.1% $(NH_4)_2SO$ and 0.06% yeast extract; and an alternative liquid medium composed of (w/v) 0.7% KH_2PO_4, 0.2% K_2HPO_4, 0.05% $MgSO_4.7H_2O$ and 0.16% $(NH_4)_2SO$. The cultures were incubated at 28°C with constant agitation at 120 rpm for 7 days. The crude extracts obtained from these cultures were filtered through filter paper (Whatman No. 1). For xylanase induction, aliquots were collected every 24 h during 7 days and used to estimate enzyme activity and protein concentration.

Enzyme assay

The xylanase activity was measured by mixing 5 µL of enzyme solution with 10 µL of oat spelt xylan (10 mg.mL^{-1}) in 50 mM sodium acetate buffer (pH 5.0) at 50°C for 30 minutes. The reaction was interrupted by the addition of 30 µL of 3,5-dinitrosalicylic acid and immediate boiling for 10 min at 97°C (Miller 1959). The release of reducing sugar was measured at 540 nm in a SpectraMax® Plus 384 (Molecular Devices, US) and xylanase activity was expressed as µmol of reducing sugar released per minute per milliliter (IU.mL^{-1}) using xylose as standard. Endoglucanase (CMCase), pectinase and mannanase activities were evaluated according to Duarte et al. (2012). Protein concentration was measured by the Bradford method (Bradford 1976), using bovine serum albumin as standard.

Enzyme purification

A crude extract sample (350 mL) obtained after *A. tamarii* cultivation in standard liquid medium was concentrated approximately 10-fold by ultrafiltration using an Amicon System (Amicon Inc., USA) with a 10-kDa cut-off point membrane. Based on the specific xylanase activity of ultrafiltrate, this sample was chosen for further purification. Aliquots (300 mL) of the ultrafiltrate were subjected to lyophilization (Freeze Dryer Liobrás, Brazil) for 48 h. The lyophilized material was re-suspended in 50 mM sodium phosphate buffer (pH 7.0) containing 150 mM NaCl and 0.02% NaN_3. Aliquots (5 mL) of re-suspended material were loaded onto Sephadex G-50 and/or Sephacryl S-100 (GE Healthcare) gel filtration systems, previously equilibrated using the same aforementioned buffer conditions. Fractions (1.5 mL) were eluted at a flow rate of 15 mL.h^{-1} and those corresponding to xylanase activity were pooled and stored at 4° for further characterization.

Enzyme characterization

For kinetic experiments, soluble oat spelt xylan was prepared as described by Filho et al. (1993). The substrate was used over a concentration range of 1-40 mg.mL^{-1}. K_M and V_{max} were estimated from the Michaelis-Menten equation with a non-linear regression data analysis program (GraphPad Prism® 6). The effects of temperature and pH were evaluated according to Duarte et al. (2012).

Dynamic Light Scattering and Tween-80 effect

Dynamic light scattering (DLS) measurements were conducted in triplicate with a Zetasizer Nanoseries (Malvern, London, UK). Conducetd at room temperature, an average of 15 acquisition scans were conducted, with total acquisition time set to 60 s. The purified and partially purified xylanases were dissolved in 50 mM sodium phosphate buffer (pH 7.0) solution. The DLS measurements were also made in the presence of 0.1% (v/v) Tween-80 under the same aforementioned conditions.

Effect of corncob autohydrolysis liquor

The autohydrolysis process was conducted 30-minute intervals, as previously described by Michelin et al. (2012). Corncob particles were decomposed to soluble compounds. The resulting liquor samples were separated from the solid by vacuum filtration and used as a liquid substrate. Two distinct assays were performed for assessment of xylanolytic activity of enzymes after incubation with corncob liquor. The first assay was carried out by incubating 5 μL of enzyme sample with 5 μL of xylan (20 $mg.mL^{-1}$) in 50 mM sodium acetate buffer (pH 5.0) and 5 μL of 10-fold diluted liquor solution. The second was performed by incubating 5 μL of enzyme sample with 5 μL of 50 mM sodium acetate buffer (pH 5.0) and 5 μL of 10-fold diluted liquor solution. The enzyme assay conditions were as described previously. The control reaction was performed by incubating 5 μL of enzyme sample with 5 μL of 50 mM sodium acetate buffer (pH 5.0) and 5 μL of xylan (20 $mg.mL^{-1}$).

Effect of phenolic compounds

Phenolic compounds at a concentration of 2 mM were individually mixed with xylanases and incubated at 50°C for 30 min, as described by Moreira et al. (2013). Enzymatic assays of xylanases in the presence of phenolic compounds were perfomed as described previously.

Statistical analysis

Analysis of experimental data for enzyme activities was analyzed with the software PAST 3.11. Data were submitted to factorial ANOVA and post hoc Tukey's Pairwise Comparisons with significance $P < 0.05$.

Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a 12% gels according to Laemmli (1970). Protein bands were silver stained according to Blum et al. (1987). A zymogram technique was adapted from Bischoff et al. (2006) for detection of xylanase activities. Replicate denaturing electrophoretic gels were co-polymerized with 1% of oat spelt xylan solution and stained with 0.1% Congo red solution for 1h at room temperature for xylanase activity. Gels were washed with a 1.0 M NaCl solution to remove excess dye and fixed with 0.5% acetic acid.

Mass Spectrometry

Proteins of interest were separated by SDS-PAGE and excised from gels. After spot picking, excised gels were washed with potassium ferricyanide and sodium thiosulphate to remove silver staining. The proteins were reduced with DTT, alkylated with iodoacetamide, and digested in-gel with trypsin, as described by Zhang et al. (2007). The resulting peptides were extracted with 1.0% (v/v) TFA, loaded onto a 600-nm AnchorChip™ (Bruker Daltonics, Germany) and air-dried at room temperature. α-cyiano-4-hydroxycinnamic acid (CHCA - $5\mu g.\mu L^{-1}$) matrix solution was mixed with the samples on the surface of an AnchorChip™ plate and subjected to MS analysis using a MALDI-TOF/TOF mass spectrometer (Autoflex II, BrukerDaltonics). The peptide mass spectrum was processed and database searches conducted against sequences of fungal proteins in UniProtKB/Swiss-Prot, taxonomy other fungi, using the software FlexControl 2.2 (Bruker Daltonics). Search parameters comprised: error tolerance for peptide mass lower than 50 ppm, one or zero missed cleavage sites for trypsin, carbamidomethylation as fixed modification, methionine oxidation and acetylation of N-terminal as variable modifications.

Results

Molecular identification

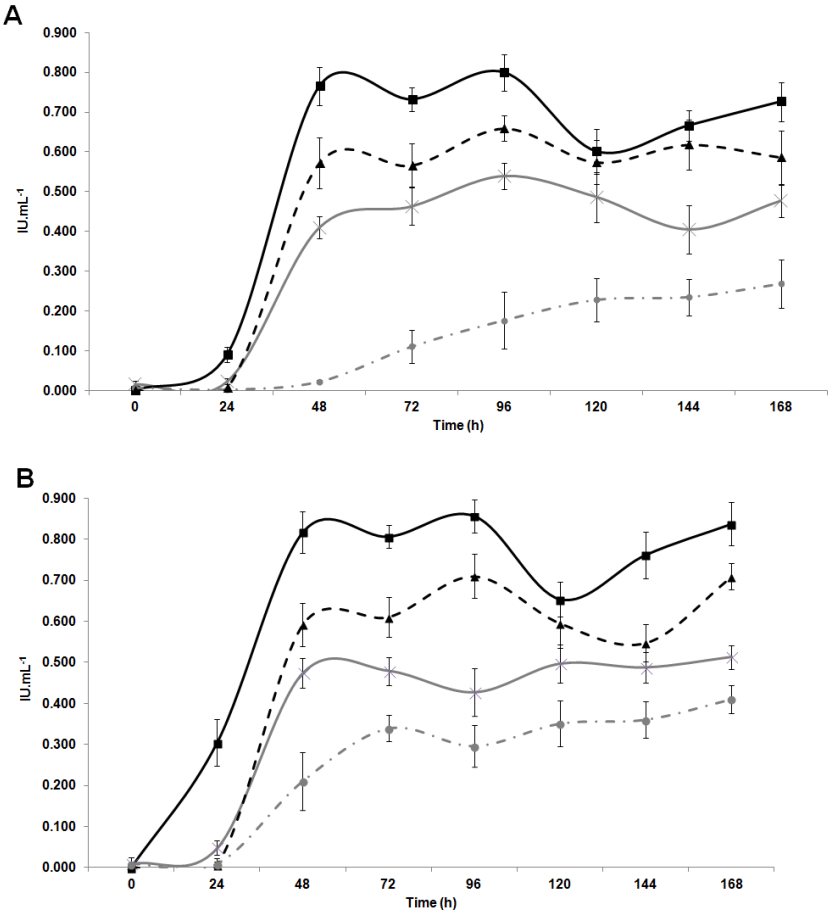


Fig. 1 – Induction profiles of xylanase activity (IU.mL^{-1}) from *A. tamarii* grown on filter powder (A) and clean cotton residue (B). Dotted lines - pretreated wastes. Continuous line - untreated wastes. Black lines - standard medium. Grey lines - alternative medium.

The rDNA ITS region was adequate for robust identification of the fungal isolate to genus level, based on significant sequence similarities with Aspergillus species. Analysis of the β-tubulin and calmodulin gene regions, which are appropriate markers for resolving closely related Aspergillus species (Pildain et al. 2008), confirmed identity with *A. tamarii*.

Growth curve profile

Fungal enzyme production profiles during growth periods can vary with the complexity of biomass and major organic sources, such as nitrogen and carbon. As expected, xylanolytic activity (IU.ml^{-1}) analysis revealed specific enzyme profiles following growth on each textile residue (Fig. 1). There were evidential differences between the curves, wherein growth on the standard medium (black lines) resulted in higher xylanolytic activity compared to activities after growth on the alternative medium (grey lines). Untreated residues (continuous line) induced more xylanolytic activity compared to pretreated residues (dotted lines).

Based on the induction profile of xylanases and protein production we established 3 days for fungal growth in pretreated residues and 4 days in untreated residues using the standard liquid medium. The xylanase activity values of tFP and tCC were 0.527 ± 0.038 IU.mL^{-1} and 0.585 ± 0.025 IU.mL^{-1}, respectively, after 3 days of growth. The xylanase activity values of FP and CC were 0.620 ± 0.045 IU.mL^{-1} and 0.705 ± 0.036 IU.mL^{-1}, respectively, after 4 days of

growth. Xylanase was most active in all cultures, followed by pectinase, CM-cellulase and mannanase activities (data not shown). The highest values for xylanase specific activities were 12.4 $IU.mg^{-1}$ for tCC; 7.821 $IU.mg^{-1}$ for FP; 4.757 $IU.mg^{-1}$ for tFP and 3.296 $IU.mg^{-1}$ for CC.

Enzyme production and purification

After ultrafiltration, the highest values of specific xylanase activities were obtained for the ultrafiltrate samples of tFP (217.17 $IU.mg^{-1}$), FP (74.5 $IU.mg^{-1}$), tCC (52.42 $IU.mg^{-1}$) and CC (42.5 $IU.mg^{-1}$). SDS-PAGE of the four ultrafiltrates revealed protein bands with molecular weights ranging from 20 to 96 kDa, while zymogram analysis showed single protein bands of 22 and 35 kDa which were confirmed with a positive stain for xylanase activity (Fig. 2).

Ultrafiltrate samples from pretreated residues were loaded onto Sephadex G-50 and from untreated residues were loaded onto Sephacryl S-100 gel filtration systems. The elution profile for both gel filtration chromatography procedures revealed two major peaks, displaying xylanase activity. Xylanases from ultrafiltrate tFP eluted from G-50 (Fig. 3) were purified with a one single-step procedure (Fig. 4) and were named Xyl-1 (35 kDa) and Xyl-2 (22 kDa). Xylanases from ultrafiltrate FP eluted from S-100 were partially purified (data not shown) and were named Xyl-3 (35 kDa) and Xyl-4 (22 kDa). Xylanases from ultrafiltrate CC eluted from S-100 were partially purified (data not shown) and were named Xyl-5 (35 kDa) and Xyl-6 (22 kDa). Finally, xylanases from ultrafiltrate tCC eluted from G-50 were partially purified (data not shown) and were named Xyl-7 (35 kDa) and Xyl-8 (22 kDa). It can be seen that Xyl-1, Xyl-3, Xyl-5 and Xyl-7 display the same molecular weight (35 kDa), as do Xyl-2, Xyl-4, Xyl-6 and Xyl-8 (22 kDa).

Peptide mass fingerprint analysis by mass spectrometry of Xyl-1 identified five peptides with a 19% sequence coverage and a matching score of 55, confirmed by the homology of Xyl-1 as an endo-β-1,4-xylanase F1 from *A. oryzae* RIB40 (reference genome strain) (Fig. 5). The predicted nominal molecular mass of 35,552 kDa for this protein was identified and confirmed by the apparent molecular weight of the purified protein on SDS-PAGE gels and zymograms.

Enzyme characterization

The purified enzymes Xyl-1 and Xyl-2 were submitted to optimal pH characterization; they presented higher activity at pH 6.0 and retained at least 40% of activity over a pH range of 3.5-9.0. Optimum temperature, K_M ($mg.mL^{-1}$) and V_{max} ($IU.mL^{-1}.s^{-1}$) values are summarized in Table 1. All xylanases retained at least 50% of their activity over the range of 30-60ºC.

To evaluate the purity and self-association tendency of the xylanases, DLS measurements were performed. The DLS-derived intensity and mass distributions for Xyl-1 without Tween-80 (Fig. 6-A) showed one peak with a molecular weight of $1.58.10^6 \pm 2.35.10^5$ kDa and size of 531.2 ± 72.93 nm. The peak was monodisperse, meaning one population of large particles, and demonstrating the self-association tendency of xylanase at pH 7.0 and concentration of 0.3 $\mu g.mL^{-1}$. When the non-ionic surfactant Tween-80 was added (Fig. 6-B), two monodispersed peaks with different mass distribution appeared. One peak appeared with the same molecular weight and size of the aggregate and another peak appeared with low intensity but higher mass distribution, with a molecular weight of 105.6 ± 23.3 kDa and 8.721 ± 1.258 nm in size, corresponding to xylanases self-assembled as a trimer. The monodisperse feature of this peak clearly demonstrates that the sample remained homogeneous, with a polidispersivity of 13.5%, indicating that just one population size is present in this condition. Similar results were found with other xylanases (data not shown), where peaks corresponding to a larger size than the average were observed, indicating the self-association tendency of these enzymes. To assess whether Tween-80 affected xylanolytic activity, an assay with the samples containing 0.1% Tween-80 was performed after DLS measurement. The results showed a slight increase of xylanase activity for all enzymes (data not shown).

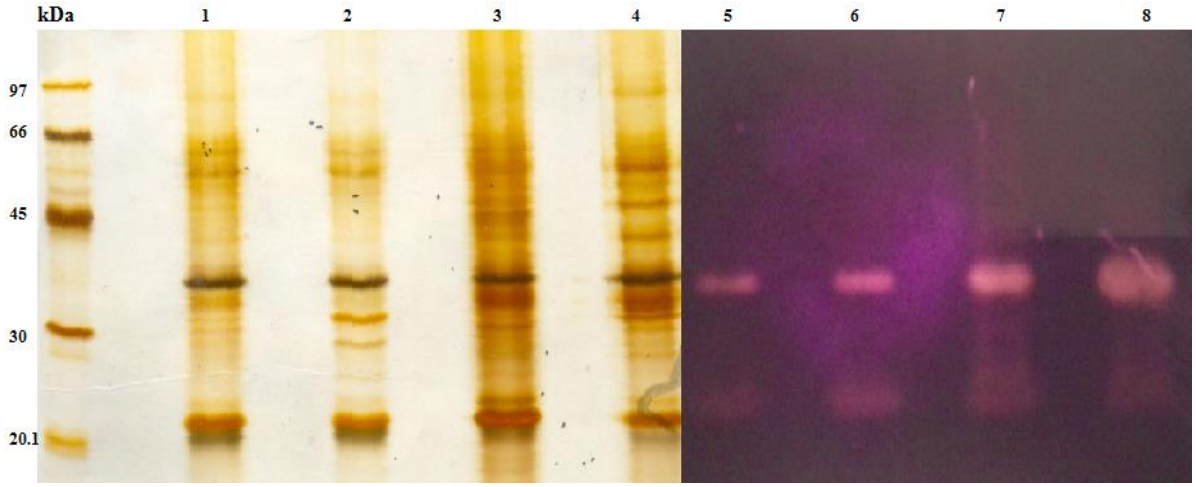


Fig. 2 – SDS-PAGE (lines 1–4) and zymogram (lines 5–8) analysis of ultrafiltrates. Lanes: 1 and 5 – ultrafiltrate FP. Lane 2 and 6 – ultrafiltrate tFP. Lane 3 and 7 – ultrafiltrate CC. Lane 4 and 8 – ultrafiltrate tCC.

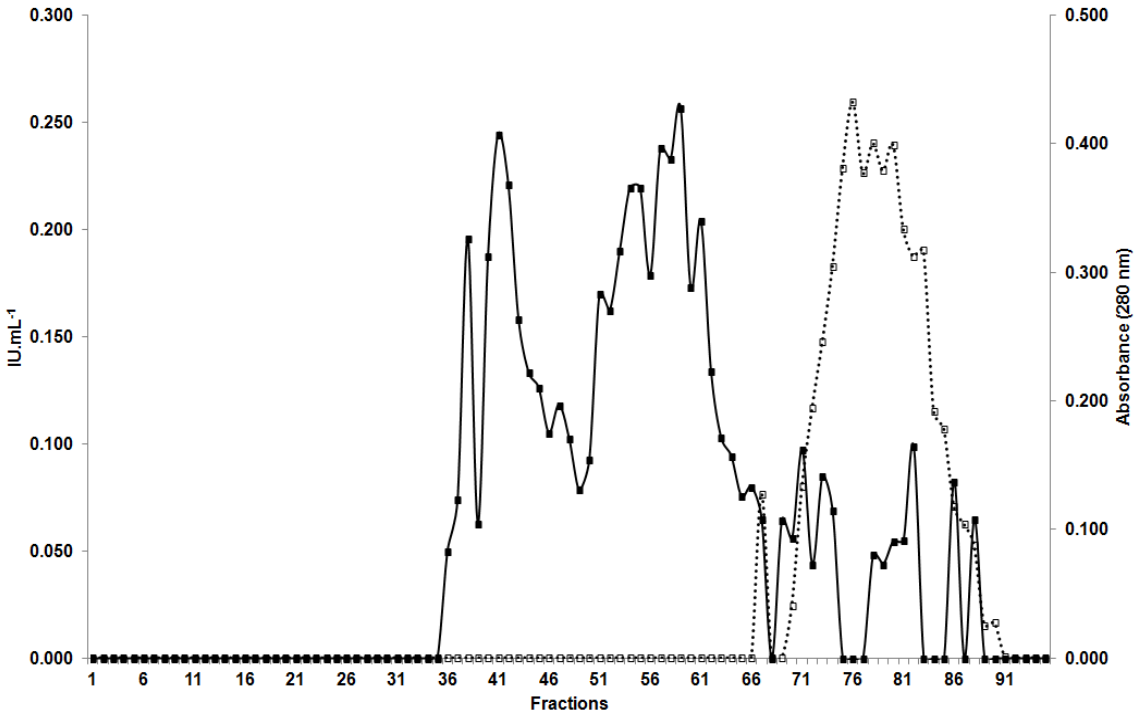


Fig. 3 – Chromatography profile of ultrafiltrate tFP in Sephadex G-50. Continuous line – xylanolytic activity ($IU.mL^{-1}$). Dotted line – UV absorbance at 280 nm.

Effect of corncob autohydrolysis liquor

Two distinct assays were performed to assess the xylanolytic activity after incubation with corncob liquor. The first assay was conducted by incubating xylanases with liquor and substrate, whilst the second was conducted by incubating xylanases with liquor and buffer replacing the substrate (Table 2). Table 2 shows that the incubation of corncob liquor with substrate increased all xylanase activities, with the exception of Xyl-5. In the absence of substrate, corncob liquor had no effect in Xyl-1 and inhibited the others xylanases.

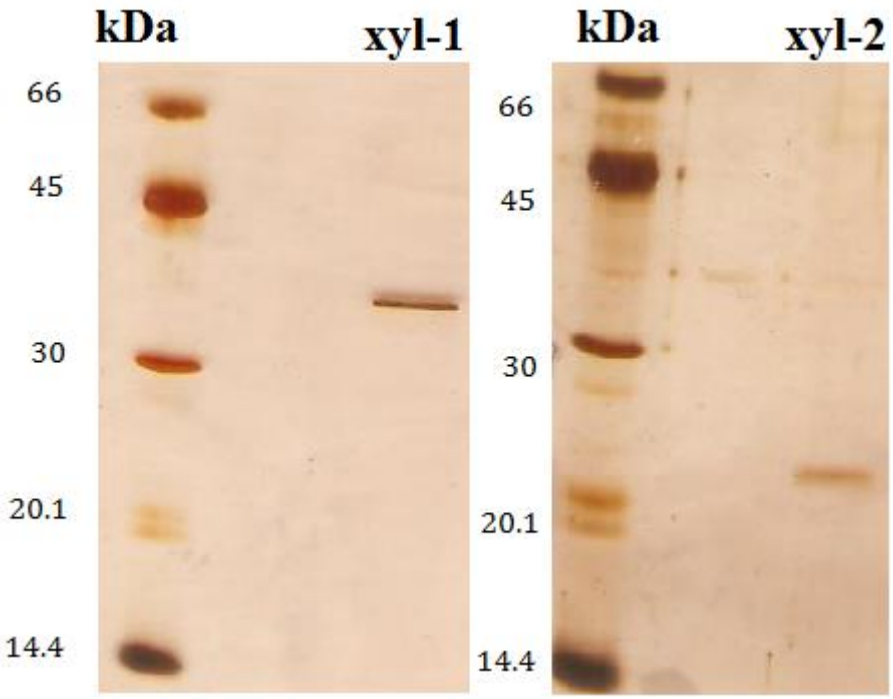
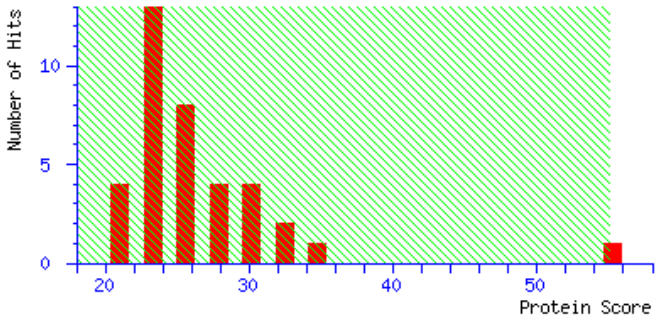


Fig. 4 – SDS-PAGE of purified fractions of tFP on Sephadex G-50 – xyl-1 and xyl-2.



Protein sequence coverage: 19%

Matched peptides shown in ***bold red***.

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  1 MVHLKALASG TLFASLASSA VISRQAAASI NDAFVAHGKK YFGTCSDQAL
 51 LQNSQNEAIV RADFGQLTPE NSMKWDALEP SQGSFSFAGA DFLADYAKTN
101 NKLVRGHTLV WHSQLPSWVQ GITDKDTLTE VIKNHITTIM QRYKGQIYAW
151 DVVNEIFDED GTLRDSVFSQ VLGEDFVRIA FETAREADPN AKLYINDYNL
201 DSADYAKTKG MVSYVKKWLD AGVPIDGIGS QSHYSANGFP VSGAKGALTA
251 LASTGVSEVA VTELDIEGAS SESYLEVVNA CLDVSSCVGI TVWGVSDKDS
301 WRSSTSPLLF DSNYQAKDAY NAIIDAL
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Start – End	Observed	Mr(expt)	Mr(calc)	ppm	M	Peptide
62 – 74	1437.6216	1436.6143	1436.6606	-32.2	0	R.ADFGQLTPENSMK.W
165 – 178	1597.6455	1596.6382	1596.7784	-87.8	0	R.DSVFSQVLGEDFVR.I
179 – 185	807.4190	806.4117	806.4286	-21.0	0	R.IAFETAR.E
193 – 207	1777.7061	1776.6989	1776.8206	-68.5	0	K.LYINDYNLDSADYAK.T
303 – 317	1657.6202	1656.6129	1656.7995	-113	0	R.SSTSPLLFDSNYQAK.D

Fig. 5 – Matching score and sequence coverage of Xyl-1 in mass spectrometry

Table 1 Biochemical characterization of xylanases.

	Molecular mass (kDa)	K_M (mg.mL^{-1})	V_{max} (IU.mL^{-1}.s^{-1})	Optimum temperature (°C)
Xyl-1	35	04.30	0.249	60
Xyl-2	22	18.92	1.103	40
Xyl-3	35	32.25	1.071	50
Xyl-4	22	10.35	0.637	50
Xyl-5	35	18.80	1.264	60
Xyl-6	22	11.12	0.202	50
Xyl-7	35	25.32	0.571	50
Xyl-8	22	37.27	1.495	50

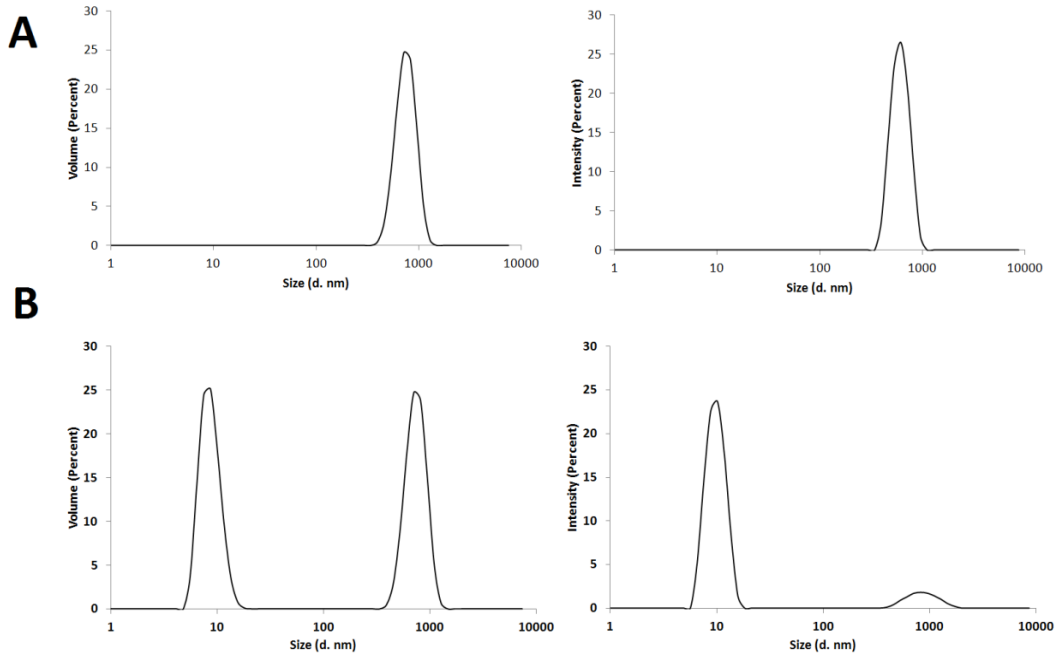

Fig. 6 – DLS measurement of Xyl-1, volume per size at left and intensity per size at right. A – Xyl-1 without 0.1% Tween-80. B – Xyl-1 with 0.1% Tween-80.

Table 2 Effect of corncob liquor and substrate on xylanase activity

	Control activity (IU.mL^{-1})	With substrate	Without substrate
		Relative activity (%)	Relative activity (%)
Xyl-1	0.100 ± 0.005	139.43 ± 0.13*	115.04 ± 0.14
Xyl-2	0.232 ± 0.016	150.70 ± 0.14*	31.16 ± 0.10*
Xyl-3	0.350 ± 0.020	124.07 ± 0.08	43.09 ± 0.10*
Xyl-4	0.300 ± 0.028	133.80 ± 0.17*	36.76 ± 0.10*
Xyl-5	0.262 ± 0.021	82.03 ± 0.17*	20.46 ± 0.15*
Xyl-6	0.087 ± 0.012	187.47 ± 0.16*	65.20 ± 0.07*
Xyl-7	0.290 ± 0.033	129.02 ± 0.30*	61.57 ± 0.46*
Xyl-8	0.127 ± 0.015	216.13 ± 0.45*	75.99 ± 0.23*

* Indicates statistical differences in Tukey's pairwise test.

Effect of phenolic compounds

The inhibitory effect of phenols was evaluated by incubating xylanases with phenolic compounds derived from the breakdown of lignocellulosic biomass and known as inhibitors of enzymatic activity. The residual xylanase activity after incubation was measured (Table 3).

Table 3 Effect of phenolic compounds on xylanase activity.

	Relative activity (%)			
	Xyl-1	Xyl-3	Xyl-5	Xyl-7
Control	100.00 ± 0.11	100.00 ± 0.01	100.00 ± 0.13	100.00 ± 0.06
Ferulic acid	151.49 ± 0.77*	171.86 ± 2.67*	135.91 ± 1.02*	137.66 ± 0.37*
ρ-Coumaric acid	70.9 ± 1.04*	112.19 ± 1.55	87.7 ± 0.92	96.55 ± 0.48
Cinnamic acid	84.68 ± 0.90	100.56 ± 0.69	95.75 ± 0.48	91.24 ± 0.32
Vanillin	92.33 ± 0.83	131.23 ± 0.38	93.41 ± 0.39	106.36 ± 0.90
4-Hydroxy-benzoic acid	93.13 ± 0.20	118.07 ± 0.07	88.81 ± 0.09	98.93 ± 0.87
Tannic acid	99.31 ± 0.76	119.19 ± 0.93	100.32 ± 0.34	96.55 ± 0.56
	Relative activity (%)			
	Xyl-2	Xyl-4	Xyl-6	Xyl-8
Control	100.00 ± 0.08	100.00 ± 0.05	100.00 ± 0.14	100.00 ± 0.12
Ferulic acid	121.74 ± 0.64*	120.52 ± 1.35*	204.56 ± 1.22*	167.85 ± 1.43*
ρ-Coumaric acid	106.7 ± 0.81	112.3 ± 0.17	127.4 ± 0.67	104.16 ± 1.49
Cinnamic acid	102.45 ± 0.60	98.51 ± 0.40	128.48 ± 1.33	102.97 ± 0.89
Vanillin	50.65 ± 0.94*	120.33 ± 3.04*	119.59 ± 1.33	85.11 ± 2.08
4-Hydroxy-benzoic acid	122.70 ± 0.64*	120.93 ± 0.54*	126.22 ± 0.55	98.21 ± 1.43
Tannic acid	92.75 ± 0.34	113.06 ± 0.57	161.08 ± 1.33*	85.71 ± 1.19

* Indicates statistical differences in Tukey's pairwise test.

It can be seen that ferulic acid significantly increased all xylanase activities. Xyl-1 was inhibited by ρ-Coumaric acid, Xyl-2 was activated by 4-Hydroxy-benzoic acid and strongly inhibited by vanillin, Xyl-4 was activated by 4-Hydroxy-benzoic acid and vanillin, and Xyl-6 was activated by tannic acid. The strongest activation effect was observed when ferulic acid was incubated with Xyl-6 (204.56 ± 1.22 % of relative activity compared with the control). The strongest inhibition effect was observed when vanillin was incubated with Xyl-2 (50.65 ± 0.94 % of relative activity compared with the control).

Discussion

Based on the induction profiles observed on both residues following 48 h of fungal growth, the enzymatic activities observed revealed that the xylanases were secreted at a constant rate, indicating continued access to the hemicellulose fibers over the cultivation timecourse. This demonstrates that these secreted enzymes in *A. tamarii* BLU37 are capable of hydrolysing biomass with high efficiency. The xylanolytic activity values observed were lower in pretreated residues when compared to untreated residues. This indicates that pretreatment was effective in reducing hemicellulose content, as with less substrate present in the biomass, there is an expected reduction in enzyme production, or a reduced induction effect. Induction effects on enzymatic activity and on saccharification have been reported in previous studies (Saykhedkar et al. 2012, Brown et al. 2014, Martins et al. 2014) and all correlate the amount of substrate with enzymatic activity. Xylanase activity profiles differed between residues in alternative and standard medium. The alternative medium, which contained only ammonium sulfate as nitrogen

source, resulted in lower enzymatic activity values than observed following growth on the standard medium, which was supplemented with yeast extract. This indicates that xylanase production may have been negatively affected by the absence of yeast extract, revealing the importance of its inclusion in a minimal medium for *A. tamarii* growth, contributing not only as a nitrogen source, but also as a source of essential vitamins and amino acids.

Biomass substrates are known to induce certain microorganisms to secrete enzyme systems and multi-enzymes with different molecular weights and with specialized functions and features in order to hydrolyze efficiently the cellulose and hemicellulose contents of the plant cell wall (Wong et al. 1988). In this study, xylanases with molecular weight greater than 10 kDa were detected in the ultrafiltrate, revealing the ability of endo-β-1,4-xylanases to change their conformation and pass through membranes with a cutoff of 10 kDa. The characterization of the purified and partially purified xylanases showed that *A. tamarii* can produce multiple forms of xylanases with similar molecular weights but with different features, explicit by different K_M and V_{max} values, and probably induced by different carbon sources and biomass composition. Although xylanase Xyl-1 was produced by *A. tamarii*, peptide mass fingerprint analysis showed a similarity with endo-β-1,4-xylanase F1 (XynF1) from *A. oryzae* RIB40, with five matched peptides, resulting in 19% coverage. Multiplicity forms of xylanase might be controlled by complex carbon sources where the fungus grows, indicating that not only a diversity of xylanase, but other intra and extra-cellular components are up-regulated by biomass composition and nutrient factors (Raman et al. 2009, Gladden et al. 2012). This diversity likely occurs so that saprophytic fungi can adapt to different lignocellulosic biomass substrates, through recognition of substrate and activation of pathway-specific transcription factors (Brown et al, 2014). Certain factors can explain this differential expression, such as growth conditions, epigenetic regulation, differential RNA processing and post-translational modification such as glycosylation, although the kind of modification that is triggered by the type of substrate and the pathways involved remain unclear (Raman et al. 2009, Gladden et al. 2012, Brown et al. 2014).

Two low molecular-weight xylanases were purified with one single chromatographic step. Optimum pH and temperature were the same as reported in previous studies that characterized similar xylanases from *A. oryzae* (Kitamoto et al. 1999, Duarte et al. 2012). Xyl-1 retained at least 40% of its activity in the pH range of 3.5-9.0, indicating its potential application in the animal feed industry, where xylanase is normally used as an additive and where high activity is required in different pH environments (Krengel et al. 1996). Another potential application is in enzymatic pulp pre-bleaching, where alkaline conditions are required throughout the process (Weerachavangkul et al. 2012).

DLS measurements with Tween-80 revealed enzymatic protein disaggregation and an increase in enzymatic activity. These results might be related to the exposure of catalytic sites after protein disaggregation, in agreement with increased enzymatic activity in the presence of Tween-80, as reported by Do et al. (2013). Tween-80 is normally used as a surfactant in the lignocellulosic biomass conversion during pretreatment and to recycle enzymes that are nonspecifically adsorbed to lignin (Van Dyk and Pletschke, 2012). An interesting application of Tween-80 would be in liquid-liquid extraction procedures in enzymatic processes, as an alternative to Triton X-114 in the micellar two-phase system, given that Tween-80 was shown to be an efficient disaggregation and non-denaturing agent.

In order to evaluate the potential industrial application of the enzymes in biomass hydrolysis, the xylanases were incubated with corncob autohydrolysis liquor. According to Michelin et al. (2012), the composition of this liquor is mainly composed of xylooligosaccharides (25.39 $g.L^{-1}$), together with other oligosaccharides (glycosaccharides and arabinosaccharides), monosaccharides (xylose, glucose, arabinose), and furfural and hydroxymethylfurfural at concentrations of 0.19 $g\text{-}L^{-1}$ and 0.77 $g.L^{-1}$, respectively. Considering that xylanases can be inhibited by the presence of xylooligosaccharides, furfural and hydroxymethylfurfural (Polizeli et al. 2005), the results showed that, despite the inhibition of

Xyl-5, the xylanases proved to be very active on xylooligosaccharides. This activation indicates that the substrate present (oat spelt xylan 2%) was probably not sufficient to saturate the catalytic sites of the enzymes, such that they were still capable of hydrolyzing more soluble substrate, demonstrating a great catalytic efficiency. Without the presence of the oat spelt xylan, the liquor proved to be an alternative substrate for Xyl-1, which showed activity when incubated in the presence of liquor. An interesting perspective for this liquor would be its use as a specific enzymatic substrate, targeting the preference of the xylanase to more deconstructed substrates, as this liquor contains hemicellulose soluble fractions of corncob. Another perspective would be as carbon source for xylanase production in liquid cultures (Michelin et al. 2012).

Although phenols from lignocellulosic biomass have been reported to inhibit enzyme activity (Kim et al. 2011) our study revealed an increase in xylanase activity in all enzymes incubated with ferulic acid, with some xylanases showing activity in the presence of one or more phenolic compounds. A similar result was found by Moreira et al. (2013) in which one purified xylanase from *A. terreus* had its activity increased when incubated with different phenolic compounds, with no loss of activity after 7 days incubation at room temperature. According to Kaya et al. (2000), the addition of phenolic compounds at low concentrations (up to 0.05%) inhibited xylanase activity of a commercial xylanase preparation (Irgazyme-40S, Ciba-Geigy Corporation, Greensboro NC). Studies have shown that inhibitory effects of phenolic compounds can occur by protein conformational changes, inducing steric inactivation (Boukari et al. 2011). The activation effect found is probably related to conformational changes associated with amino acid residues involved in maintaining the integrity of the active sites or in binding and/or hydrolysis of the substrate in the vicinity of the active site (Moreira et al. 2015).

The xylanases studied here demonstrate that their productivity and activity is related to nutrient uptake by the saprophytic fungus. Additionally, the different biochemical features of the multiple forms of xylanases could indicate a direct correlation with the biomass that induces production. Typically, xylan present in plant cell wall limits access to cellulose more directly than lignin; thereby xylan is considered as the major recalcitrant polysaccharide of the plant cell wall (Selig et al. 2009). Based on this, these multiple forms of xylanases with resistance to phenolic compounds show potential for application in second generation bioethanol industries, given that following biomass pretreatment the enzymes could support the presence of soluble lignin and hydrolyze xylan, exposing the cellulose surface to further attack. These enzymes also tolerate higher levels of soluble xylose during hydrolysis. In conclusion, *A. tamarii* BLU37 demonstrated considerable potential as a fungal strain for application in the second generation bioethanol industry.

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