



Research article

Xylanase and β -xylosidase from *Penicillium janczewskii*: Purification, characterization and hydrolysis of substrates

César Rafael Fanchini Terrasan ^a, José Manuel Guisan ^b, Eleonora Cano Carmona ^{a,*}^a Biochemistry and Microbiology Department, Biosciences Institute, Univ Estadual Paulista – UNESP, PO Box 199, Av. 24 A, no. 1515, Bela Vista, 13506-900 Rio Claro, SP, Brazil^b Departamento de Biocatálisis, Instituto de Catálisis, CSIC (Consejo Superior de Investigaciones Científicas), Campus Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain

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ABSTRACT

Background: Xylanases and β -D-xylosidases are the most important enzymes responsible for the degradation of xylan, the second main constituent of plant cell walls.

Results: In this study, the main extracellular xylanase (XYL I) and β -xylosidase (BXYL I) from the fungus *Penicillium janczewskii* were purified, characterized and applied for the hydrolysis of different substrates. Their molecular weights under denaturing and non-denaturing conditions were, respectively, 30.4 and 23.6 kDa for XYL I, and 100 and 200 kDa for BXYL I, indicating that the latter is homodimeric. XYL I is highly glycosylated (78%) with optimal activity in pH 6.0 at 65°C, while BXYL I presented lower sugar content (10.5%) and optimal activity in pH 5.0 at 75°C. The half-lives of XYL I at 55, 60 and 65°C were 125, 16 and 6 min, respectively. At 60°C, BXYL I retained almost 100% of the activity after 6 h. NH_4^+ , Na^+ , DTT and β -mercaptoethanol stimulated XYL I, while activation of BXYL I was not observed. Interestingly, XYL I was only partially inhibited by Hg^{2+} , while BXYL I was completely inhibited. Xylobiose, xylotriose and larger xylooligosaccharides were the main products from xylan hydrolysis by XYL I. BXYL I hydrolyzed xylobiose and larger xylooligosaccharides with no activity against xylans.

Conclusion: The enzymes act synergistically in the degradation of xylans, and present industrial characteristics especially in relation to optimal activity at high temperatures, prolonged stability of BXYL I at 60°C, and stability of XYL I in wide pH range.

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1. Introduction

Xylan is the second most common biopolymer in plant cell walls and one of the main hemicelluloses, consisting of a backbone of β -1,4-linked D-xylopyranosyl residues substituted at varying degrees by side groups such as glucopyranosyl, 4-O-methyl-D-glucuronopyranosyl, α -L-arabinofuranosyl, acetyl, feruloyl and p-coumaroyl residues [1]. The complete hydrolysis of xylan requires the synergistic action of various enzymes, known as a xylanolytic system [2]. Endo- β -1,4-xylanase (E.C.3.2.1.8) and β -D-xylosidase (E.C.3.2.1.37) are the most important enzymes responsible for the degradation of the polymer main chain. Typically, xylanases cleave the internal β (1 \rightarrow 4) bonds in the xylan backbone, liberating different chain-length-substituted xylooligosaccharides, and β -xylosidases are exoglycosidases that release smaller xylooligosaccharides and xylose from non-reducing ends of these xylooligosaccharides. β -Xylosidases, in fact, conduct the greatest work load in terms of glycosidic bonds

cleaved, and also alleviate inhibition by product of xylanases, making its performance of crucial importance [3]. α -L-Arabinofuranosidase (E.C.3.2.1.39), acetyl xylan esterase (E.C.3.1.1.72) and ferulic or p-coumaric acid esterase (E.C.3.2.1.73) are side chain-cleaving enzymes, known as accessory enzymes [4]. They are important since side chains are recognized by different xylanases and also the degree of substitution in the polymer will influence the xylan hydrolysis [5].

The importance in xylan degrading enzymes has increased over the last decades due to their application at different industries. Among different microorganisms, filamentous fungi are remarkably attractive because their enzymes are usually secreted into the medium and the producing levels are superior to those found in yeasts and bacteria [6]. These enzymes have great potential for applications in the bioconversion of lignocellulose to sugars, fuel ethanol and chemicals. Xylooligosaccharide is the only nutraceutical that can be produced from lignocellulose biomass. Their consumption selectively stimulates beneficial gut microflora (prebiotic activity) and presents positive effect on reduction of blood glucose and cholesterol, reduction of pro-carcinogenic enzymes, enhancement of mineral absorption and stimulation of immune system [7]. Other target applications of these enzymes are for improvement of animal nutrition, clarification of

* Corresponding author.

E-mail address: ecarmona@rc.unesp.br (E.C. Carmona).

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Nomenclature

X ₁	xylose
X ₂	xylobiose
X ₃	xylotriose
X ₄	xylotetraose
X ₅	xylopentaose
X ₆	xylohexaose
pNPX	<i>p</i> -nitrophenyl β-D-xylopyranoside
oNPX	<i>o</i> -nitrophenyl β-D-xylopyranoside
pNPAr	<i>p</i> -nitrophenyl α-L-arabinofuranoside
pNPGal	<i>p</i> -nitrophenyl α-D-galactopyranoside
pNPGlu	<i>p</i> -nitrophenyl β-D-glucopyranoside.

juices and wines, biobleaching in the pulp and paper industries and processing of fabrics [8,9,10].

Penicillium janczewskii Zalesky is an anamorph and filamentous specie of the *Penicillium* genus, consisting in a widespread soil fungus. *P. janczewskii* CRM 1348 was isolated from soil of the Brazilian Rainforest [11] and previously characterized as good producer of extracellular xylanase, β-xylosidase and α-L-arabinofuranosidase in solid [12] and liquid medium without producing cellulases [13,14]. The aim of the present study was to purify the main xylanase and a β-xylosidase produced by this fungus. Besides, the purified enzymes were biochemically characterized and applied for the hydrolysis of xylans and xylooligosaccharides, since the knowledge about their characteristics is essential for future application in bioprocesses.

2. Materials and methods

2.1. Microorganism and growth conditions

P. janczewskii (CMR 1348) is deposited in The Central of Microbial Resources, CMR-UNESP, Brazil. The fungal strain was routinely maintained on Vogel medium [15] with 1.5% (w/v) wheat bran. For inoculum production, cultures were prepared in the same medium with 1.5% (w/v) glucose and incubated for seven days at 28°C. Conidia were harvested and suspended in sterile distilled water; the concentration was adjusted to 10⁷ per mL and a volume of one milliliter of this suspension was used to inoculate the liquid medium for enzyme production. Cultures were prepared in Erlenmeyer flasks (125 mL) containing 25 mL of Vogel medium supplemented with 1% (w/v) oat spelt xylan (Sigma). The initial pH was adjusted to 6.5 and 5.0 for the production of xylanase and β-xylosidase, respectively. Cultivation was carried out in stationary condition at 30°C for 7 d and at 25°C for 5 d for the production of xylanase and β-xylosidase, respectively [16]. Xylanase and β-xylosidase production corresponded to 29.8 and 0.2 U/mL, respectively; and the final pH of the culture medium to the values of 7.2 and 6.3, respectively. The mycelium was vacuum filtered, the culture filtrate was centrifuged (10,000 × g, 15 min, 4°C) and the supernatant was used as a source of extracellular proteins.

2.2. Enzymes assays

Xylanase activity was determined according to Bailey et al. [17] with 1% (w/v) birchwood xylan (Sigma) prepared in 0.05 M sodium acetate buffer pH 5.0 or 0.05 M imidazole buffer pH 6.0 and appropriately diluted enzyme solution. Reducing sugars were quantified with DNS acid reagent [18]. β-Xylosidase activity was determined in a reaction mixture containing 0.25% (w/v) *p*-nitrophenyl β-D-xylopyranoside (Sigma) in McIlvaine buffer pH 4.0 or 5.0 and appropriately diluted enzyme solution. Reaction was stopped by the addition of a saturated sodium tetraborate solution and the absorbance was measured at

405 nm [19]. One unit of activity was defined as the amount of enzyme required to release 1 μmol of product equivalent per min in the assay conditions. Specific activities were expressed as enzyme units per milligram of protein.

2.3. Determination of protein

Protein was determined by the Lowry Method [20], using bovine serum albumin as standard.

2.4. Purification of enzymes

For the xylanase purification, the crude enzyme preparation was dialyzed against 0.05 M imidazole buffer pH 6.0 (6 h, 3 changes, 4°C) and chromatographed in an anionic exchange DEAE Sephadex A-50 column (Aldrich, 1.4 × 17.0 cm) equilibrated in the same buffer. Elution of adsorbed proteins was carried out with a linear 0.0–1.0 M NaCl gradient with the same buffer, 1.2 mL/min flow rate. Fractions (3.5 mL) with xylanase activity were pooled, dialyzed against 0.05 M ammonium acetate buffer pH 6.8 (6 h, 3 changes, 4°C), frozen (–20°C), and lyophilized. The sample was then re-suspended in 4.0 mL of this buffer and applied to size exclusion chromatography on a Sephadex G-75 column (GE Healthcare, 1.25 × 64.0 cm) equilibrated and eluted in 0.05 M ammonium acetate buffer pH 6.8, at 0.3 mL/min flow rate. The 3.0 mL fractions with xylanase activity were pooled.

For the β-xylosidase purification, the crude enzyme preparation was precipitated with ammonium sulfate up to 55% saturation. Precipitated proteins were removed by centrifugation (10,000 × g, 30 min, 4°C) and the supernatant was dialyzed against 0.05 M sodium acetate buffer pH 4.5 (6 h, 3 changes, 4°C). After dialysis, the sample was chromatographed in a cationic exchange CM Sephadex C-50 column (Aldrich, 1.4 × 17.0 cm) equilibrated with the same buffer. Adsorbed proteins were eluted at 1.2 mL/min flow rate with a linear pH gradient from pH 4.5 to 5.7 of 0.05 M sodium acetate buffer. The 3.5 mL fractions with β-xylosidase activity were pooled and presented pH 5.3. Ammonium sulfate was added to this sample (1.5 M final concentration) and then it was applied to a hydrophobic interaction Hiprep™ 16/10 Phenyl Sepharose FF (low sub) column (GE Healthcare, 1.6 × 10.0 cm), previously equilibrated in 0.05 M sodium acetate buffer pH 5.3. Adsorbed proteins were eluted at 5 mL/min flow rate with a linear 1.5–0.5 M ammonium sulfate descending gradient in the same buffer. The 3.0 mL fractions with β-xylosidase activity were pooled, dialyzed (6 h, 3 changes, 4°C) against 0.05 M ammonium acetate buffer pH 6.8, frozen (–20°C), and lyophilized. The sample was then re-suspended in 4.0 mL of this buffer and applied to a size exclusion Sephadex G-200 column (GE Healthcare, 1.25 × 64.0 cm), equilibrated and eluted with 0.05 M ammonium acetate buffer pH 6.8, at 0.3 mL/min flow rate. The 3.0 mL fractions with β-xylosidase activity were pooled.

During all chromatographic steps, proteins were followed by reading absorbance at 280 nm and the enzyme activities were measured, as previously described. A sample of each step-pooled fractions was subjected to electrophoresis. All purification procedures were carried out at 4°C.

2.5. Electrophoresis

SDS-PAGE was performed in a Miniprotein II Cell (BioRad) using 8–18% (w/v) gradient gels [21]. The resolved protein bands were visualized after staining with 0.1% (w/v) coomassie brilliant blue R-250 dissolved in methanol, acetic acid and distilled water (4:1:5, v/v/v).

2.6. Characterization of enzymes

2.6.1. Determination of molecular weight (MW)

Mixtures of standard proteins (Sigma) were used to plot the standard curve log of MW against relative mobility on SDS-PAGE.

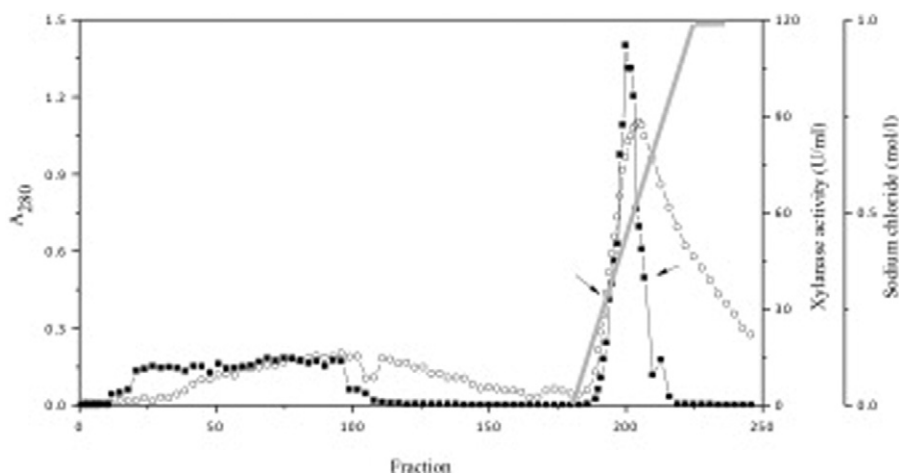


Fig. 1. Elution profile of xylanases from *P. janczewskii* in DEAE Sephadex A-50. Conditions: 0.05 M imidazole buffer pH 6.0; elution with a linear NaCl gradient in the same buffer; flow rate 1.2 mL/min; fraction 3.5 mL (○) A_{280} ; (■) xylanase activity (U/mL); (—) NaCl concentration (mol/L). Arrows indicate the pooled fractions.

Bovine serum albumin (66 kDa), glutamic dehydrogenase (55 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa) and trypsin inhibitor (20.1 kDa) (Sigma) were used for xylanase gels. β -Galactosidase (116 kDa), phosphorylase b (97 kDa), bovine serum albumin (66 kDa), glutamic dehydrogenase (55 kDa), ovalbumin (45 kDa) and glyceraldehyde 3-phosphate dehydrogenase (36 kDa) were used for β -xylosidase gels.

The MW by gel filtration was estimated by plotting log of MW of the standards against the ratio between elution volumes of the standards and the void volume (V_0), estimated using blue dextran. Ribonuclease (15.4 kDa), chymotrypsin (25 kDa), ovalbumin (43 kDa) and bovine serum albumin (66 kDa) were used for xylanase, and cytochrome c (12.4 kDa), carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa) and β -amylase (200 kDa) for β -xylosidase.

2.6.2. Determination of carbohydrate concentration

Total carbohydrate was determined by the phenol-sulfuric acid method [22], with glucose as standard. Absorbance was measured at 495 nm.

2.6.3. Optima pH and temperature, thermal and pH stability

Optimum pH values were determined by assaying xylanase activity at 65°C in pH from 1.6 to 10.0, with the following buffers:

Sorensen (pH 1.6–3.5), 0.05 M sodium acetate (pH 3.7–5.5), 0.05 M imidazole (pH 6.0–7.0), 0.05 M Tris–HCl (pH 7.0–9.0) and Sorensen (pH 9.0–10.0); and β -xylosidase activity at 75°C in pH from 2.5 to 7.0 with McIlvaine buffer. Optimum temperatures were determined by assaying xylanase activity at temperatures from 20 to 70°C in 0.05 M imidazole buffer pH 6.0, and β -xylosidase activity from 60 to 90°C with McIlvaine buffer pH 5.0.

The pH stability was determined by assaying residual activity after incubation of purified enzymes for 24 h at 4°C. Diluted (1:2 v/v) xylanase samples were incubated with the following buffers: Sorensen (pH 1.6–3.5), 0.05 M sodium acetate (pH 3.7–5.5), 0.05 M imidazole (pH 6.0–7.0), 0.05 M Tris–HCl (pH 7.0–9.0) and Sorensen (pH 9.0–10.0). Diluted (1:2 v/v) β -xylosidase samples were incubated with McIlvaine buffer (pH 2.5–8.0) and 0.05 M glycine–NaOH buffer (pH 8.6–10.5). Thermal stability was determined by assaying the remaining activity after incubation of each purified enzyme without substrate at different temperatures for different periods. Xylanase was incubated at 55, 60 and 65°C, and β -xylosidase was incubated at 60, 70 and 75°C.

2.6.4. Effect of substances

The influence of metallic ions and other chemical compounds on the activity of the purified enzymes was evaluated by adding these substances to the enzymatic reactions at 2 and 10 mM final concentrations. Activities were assayed at standard conditions and the results were expressed in relation to the control (without any substance).

2.6.5. Specificity of enzymes

Xylanase specificity was verified by assaying the activity against 1% (w/v) oat spelts xylan, birchwood xylan, beechwood xylan, carboxymethyl cellulose (CMC) and Avicel® prepared in 0.05 M imidazole buffer pH 6.0 at 65°C. β -Xylosidase specificity was verified by assaying the activity with 0.04 M o-nitrophenyl β -D-xylopyranoside (oNPPX), p-nitrophenyl α -L-arabinofuranoside (pNPArA), p-nitrophenyl α -D-galactopyranoside (pNPGal) and p-nitrophenyl β -D-glucopyranoside (pNPGlu) in McIlvaine buffer pH 5.0 at 75°C.

2.6.6. Analysis of hydrolytic products of the enzymes

Birchwood, beechwood and oat spelts xyans (Sigma, 98% purity) were prepared at 4% (w/v) initial concentration and diluted at 60°C under magnetic stirring in pH 5.0. After 1 h, the suspensions were centrifuged (10,000 \times g, 30 min.) to remove insoluble xylan. Each soluble substrate was then individually incubated with the purified xylanase (2.8 U) and β -xylosidase (0.01 U) either alone or in

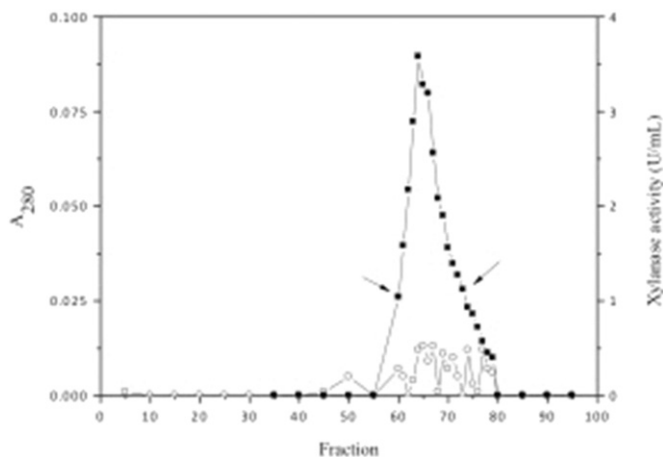


Fig. 2. Elution profile of XYL I from *P. janczewskii* in Sephadex G-75. Conditions: 0.05 M ammonium acetate buffer pH 6.8; flow rate 0.3 mL/min; fraction 3.0 mL (○) A_{280} ; (■) xylanase activity (U/mL). Arrows indicate the pooled fractions.

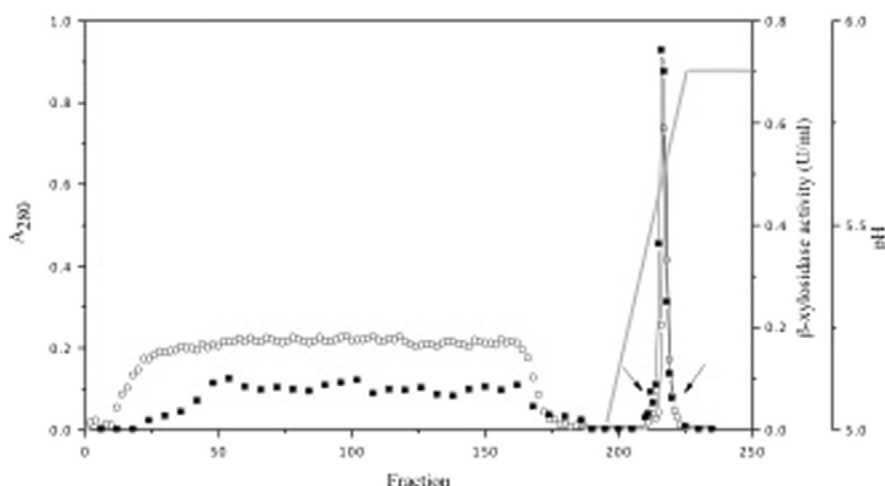


Fig. 3. Elution profile of BXYL I from *P. janczewskii* in CM Sephadex C-50. Conditions: 0.05 M sodium acetate buffer pH 5.0; elution with a linear pH gradient in the same buffer; flow rate 1.2 mL/min; fraction 3.5 mL (○) A_{280} ; (■) β -xylosidase activity (U/mL); (—) pH gradient. Arrows indicate the pooled fractions.

combination (15 mL reaction volume), under mild agitation at 30°C. Samples were taken after 24 h and reactions were stopped by boiling the samples for 10 min. Control samples ($t = 0$ and 24 h) corresponded to each substrate without the enzymes. Samples were then centrifuged ($12,000 \times g$, 15 min), filtered (0.2 μ m filters) and the adequately diluted supernatant was analyzed by HPLC.

The purified β -xylosidase was individually incubated with 0.5 g L⁻¹ solutions of xylobiose, xylotriose, xylotetraose, xylopentaose and xylohexaose (Megazyme, Wicklow, Ireland) in pH 5.0 at 50°C. Samples were taken at several interval times and hydrolysis reactions were stopped by boiling the samples for 10 min. Samples were then filtered (0.2 μ m filters), adequately diluted and analyzed by HPLC.

High-performance liquid chromatography (HPLC) was coupled with low-temperature evaporative light-scattering detection LT-ELSD using a detector Sedex75 (Sedere, Orleans, France). Analyses were carried out at 80°C on a Rezec R50-oligosaccharide Ag⁺ 4% column (200 \times 10 mm) (Phenomenex, Torrance, USA). The elution was carried out with MilliQ ultrapure apyrogenic water at 0.3 mL min⁻¹ flow rate using a TSP P100 pump and AS3000 autosampler Spectra Series (Thermo Electron Co., San Jose, US). Data acquisition and processing was performed using the Chromquest 4.1 software (Thermo Electron Co., San Jose, USA). Xylose, xylobiose, xylotriose, xylotetraose, xylopentaose and xylohexaose were used as standards.

2.6.7. Enzyme kinetics

Xylanase activity was assayed with oat spelts and birchwood xylan at different concentrations in 0.05 M imidazole buffer pH 6.0 at 65°C. β -Xylosidase activity was assayed with pNPX at different concentrations in McIlvaine buffer pH 5.0 at 75°C. The Michaelis–Menten constant (K_m) and maximum reaction velocity (V_{max}) were estimated from the Lineweaver–Burk plot [23]. Xylose inhibition of BXYL I was determined by carrying out enzyme assays with 0.68, 1.35, 2.7 and 5.4 mM pNPX varying amounts of xylose (1.8, 4.5, 9 and 18 mM final concentration) in McIlvaine buffer pH 5.0 at 75°C. The K_i was calculated from a Dixon plot.

3. Results and discussion

3.1. Purification of the main xylanase and β -xylosidase from *P. janczewskii*

Classical purification techniques were applicable to purify the main xylanase and β -xylosidase produced by *P. janczewskii*. These extracellular enzymes were purified from the crude extract obtained of stationary liquid cultures with oat spelts xylan and slightly different cultivation conditions of pH and temperature [16].

The xylanase was purified by a sequence of ion exchange and size exclusion chromatographic steps. After ion-exchange chromatography

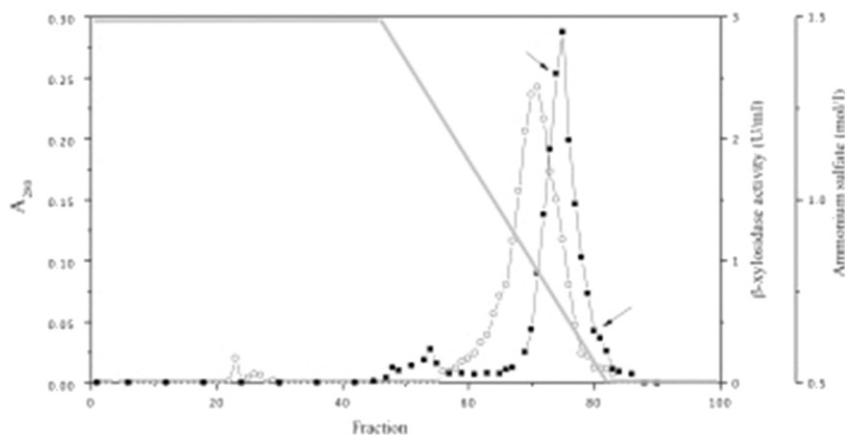


Fig. 4. Elution profile of BXYL I from *P. janczewskii* in phenyl agarose. Conditions: 0.05 M sodium acetate buffer pH 5.3; elution with a linear salt gradient in the same buffer; flow rate 5 mL/min; fraction 3.0 mL (○) A_{280} ; (■) β -xylosidase activity (U/mL); (—) Ammonium sulfate concentration (mol/l). Arrows indicate the pooled fractions.

(Fig. 1), xylanase activity was observed in both adsorbed and non-adsorbed fractions, with most of the activity observed in the non-adsorbed fraction (90%) that was named XYL I, the main xylanase from *P. janczewskii*.

Fractions were pooled, frozen, concentrated by lyophilization and subsequently submitted to molecular exclusion chromatography, resulting in only one protein peak with xylanase activity (Fig. 2).

The purification of the *P. janczewskii* β -xylosidase required four sequential steps i.e. ammonium sulfate precipitation, ion exchange, hydrophobic interaction and size exclusion chromatographies. Previous precipitation studies showed that most of the activity (66%) remained in the supernatant of 55% ammonium sulfate saturation (not shown), and the enzyme present in this supernatant was named BXYL I, the main β -xylosidase produced by *P. janczewskii*. The dialyzed supernatant was loaded into an ion exchange column and could only be successfully desorbed with a pH change. Desorbed fractions with β -xylosidase activity were pooled, presenting pH 5.3 (Fig. 3).

Ammonium sulfate was added to this sample and it was subjected to a hydrophobic interaction column. Protein desorption was carried out with a descending ammonium sulfate linear gradient (Fig. 4).

Fractions with β -xylosidase activity were pooled, concentrated by lyophilization and applied to size exclusion chromatography, resulting in the elution of only one peak with β -xylosidase activity (Fig. 5).

After the purification steps, samples of both enzymes showed electrophoretic homogeneity (Fig. 6a and Fig. 6b).

XYL I exhibited specific activity of 809.8 U mg prot.⁻¹ and it was 5.4-fold purified with 28.4% recovery. BXYL I presented specific activity of 1060.0 U mg prot.⁻¹; it was highly purified (557.9-fold) and the purification presented 6.2% recovery (Table 1). The two last purification steps presented lower yield, although they were essential to separate a protein similar to BXYL I (not shown).

The presence of xylanase activity both in the adsorbed and non-adsorbed fractions in the ion exchange chromatography, as well as the presence of β -xylosidase activity in two distinct precipitation ranges indicated the production of at least two isoforms of these enzymes. Multiplicity is an adaptive strategy found by microorganisms to achieve more effective hydrolysis of heterogeneous substrates in nature, an environment that is prone to be altered during their growth [24,25]. Thus, each produced enzyme probably fits and degrades a substrate of particular arrangement and environment at a given time [26]. The occurrence of multiples β -xylosidases is less related than the multiplicity of xylanases. Among *Penicillium* strains, three xylanases from *Penicillium capsulatum* [27,28], and two xylanases

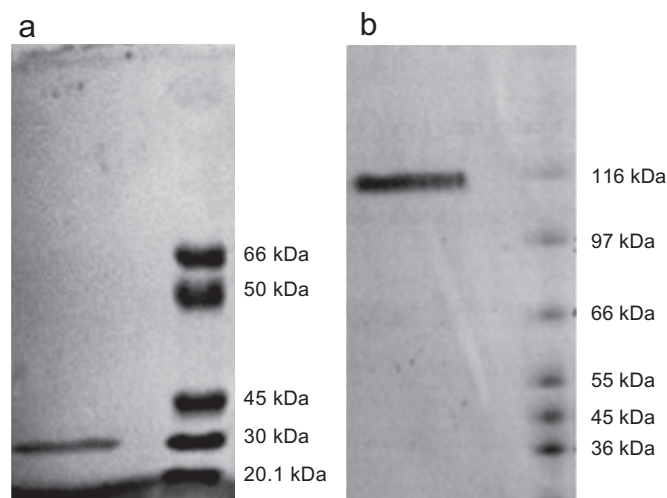


Fig. 6. SDS-PAGE (8–18%) profiles of standard proteins and purified XYL I (a) and BXYL I (b) from *P. janczewskii*. (a): Left: XYL I; right: albumin (66 kDa), glutamic dehydrogenase (55 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa). (b) Left: BXYL I; right: β -galactosidase (116 kDa), phosphorylase b (97 kDa), albumin (66 kDa), glutamic dehydrogenase (55 kDa), ovalbumin (45 kDa) and glyceraldehyde 3-phosphate dehydrogenase (36 kDa). Proteins were stained with coomassie brilliant blue R-250.

from *Penicillium purpurogenum* were purified [29]. Multiplicity of β -xylosidases was reported for *Penicillium herquei* [30] and *Penicillium wortmanni* [31].

3.2. Characterization of the main xylanase and β -xylosidase from *P. janczewskii*

The MW of XYL I estimated by SDS-PAGE and gel filtration were 30.4 and 23.6 kDa, respectively; and the MW of BXYL I estimated by these methods were 100 and 200 kDa, respectively. The results indicate BXYL I is a dimer of identical subunits, since only a single band was observed by SDS-PAGE. Similarly, homodimeric microbial β -xylosidases were observed in *Neocallimastix frontalis* [32], *Aspergillus pulverulentus* [33] and *Aspergillus awamori* X-100 [34], while other β -xylosidases correspond to heterodimers as those from *Humicola insolens* [35] and *Penicillium sclerotiorum* [36].

Xylanase activity was observed from pH 6.0 to 7.0, with optimum in pH 6.0, while elevated β -xylosidase activity was verified in more acid pH in the range from 3.0 to 5.0, with maximum in pH 5.0 (Fig. 7a). These values are similar to other *Penicillium* spp. xylanases [37] and β -xylosidases [38]. XYL I was optimally active at 65°C and BXYL I presented elevated activity between 70 and 80°C, with optimum at

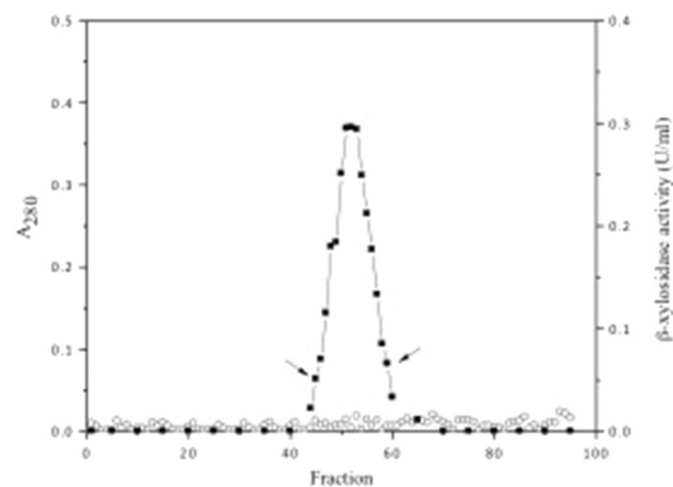


Fig. 5. Elution profile of BXYL I from *P. janczewskii* in Sephadex G-200. Conditions: 0.05 M ammonium acetate buffer pH 6.8; flow rate 0.3 mL/min; fraction 3.0 mL. (○) A₂₈₀; (■) β -xylosidase activity (U/mL). Arrows indicate the pooled fractions.

Table 1
Purification of XYL I and BXYL I from *P. janczewskii*.

Step	Total activity (U)	Total protein (mg)	Specific activity (U mg prot. ⁻¹)	Yield (%)	Purification
XYL I					
Crude filtrate	9967.8	66.0	151.0	100.0	1.0
DEAE Sephadex A-50	8966.3	25.5	351.6	90.0	2.3
Sephadex G-75	2834.2	3.5	809.8	28.4	5.4
BXYL I					
Crude filtrate	172.2	93.3	1.9	100.0	1.0
Supernatant 55% sat. ammonium sulfate	110.4	62.1	1.8	64.1	1.0
CM Sephadex C-50	65.4	29.1	2.3	38.0	1.2
Phenyl agarose	27.2	0.9	30.2	15.8	15.9
Sephadex G-200	10.6	1 × 10 ⁻²	1060.0	6.2	557.9

Xylanase activity was assayed in 0.05 M sodium acetate buffer pH 5.5 at 50°C; β -xylosidase activity was assayed in McIlvaine buffer pH 5.0 at 75°C.

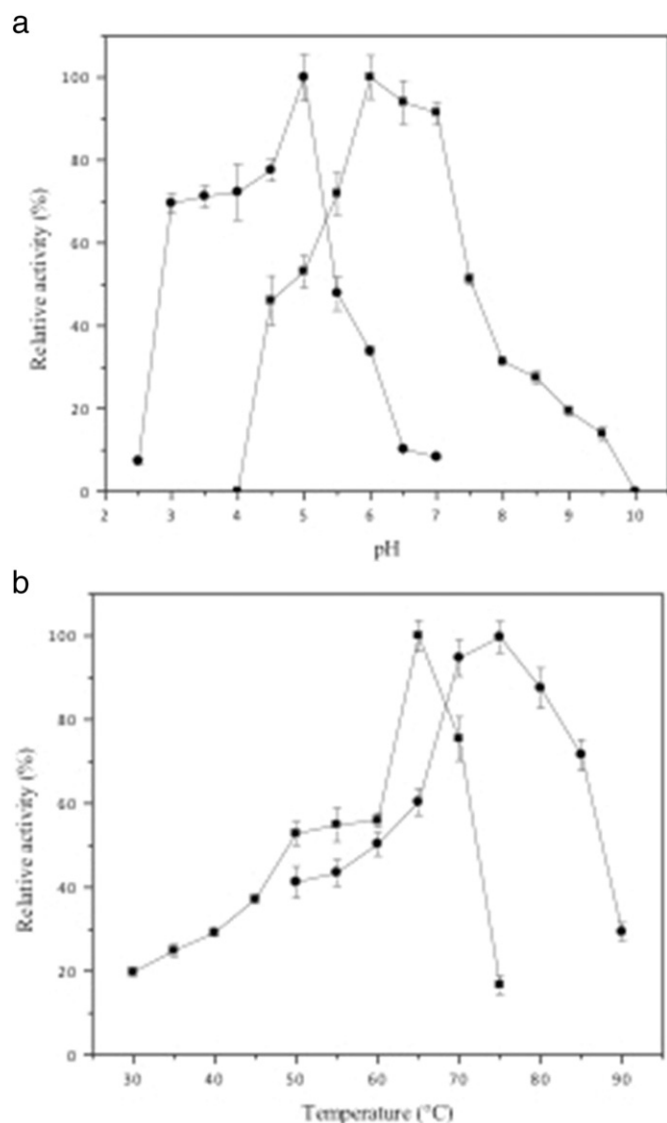


Fig. 7. Influence of pH (a) and temperature (a) on the activity of XYL I and BXYL I from *P. janczewskii*. (■) Relative xylanase activity (%); (●) Relative β -xylosidase activity (%). Conditions: (a) Xylanase activity was assayed in 0.05 M sodium acetate buffer pH 3.7 to 5.5; 0.05 M imidazole buffer pH 6.0, 6.5 and 7.0, 0.05 M Tris-HCl buffer pH 7.5, 8.0 and 8.5, and Sorensen buffer pH 9.0, 9.5 and 10.0, at 50°C. β -Xylosidase activity was assayed in Mcllvaine buffer pH from 2.5 to 7.0, at 75°C. (b) Xylanase activity was assayed in 0.05 M imidazole buffer pH 6.0. β -Xylosidase activity was assayed in Mcllvaine buffer pH 5.0.

75°C (Fig. 7b). Activity at high temperatures is interesting considering the fungus is mesophilic and can be very important for a future application in processes that are carried out at high temperatures, such as biobleaching of pulps in the pulp and paper industry, bioprocessing of fabrics and bioconversion of xylan into value added products. Similarly, the xylanase from *Penicillium simplicissimum* presented optimal activity at 67°C [39]. Nevertheless, activity at high temperatures was not described for β -xylosidases from other *Penicillium* species, only for those from *Aspergillus* strains such as *Aspergillus phoenicis* [40], *Aspergillus japonicus* [41] and *Aspergillus fumigatus* [42] with optimum between 70 and 75°C.

Apart from temperature and pH optima, thermal and pH stability constitute important properties when studying the industrial importance of an enzyme. XYL I was stable in pH from 2.5 to 10.0, retaining more than 50% of activity in this pH range. BXYL I was stable in pH from 3.5 to 8.5, retaining more than 70% of the activity in this pH range (not shown). The broad pH stability range is very

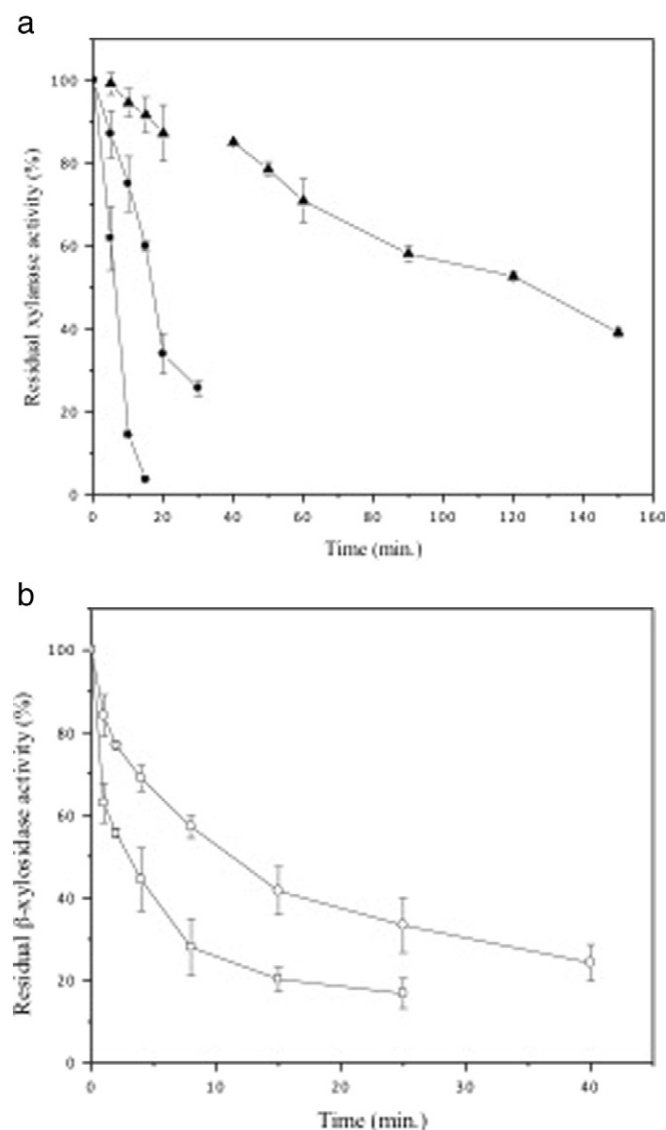


Fig. 8. Thermal stability of XYL I (a) and BXYL I (b) from *P. janczewskii*. Conditions: (a) XYL I was incubated without substrate at (▲) 55, (●) 60 and (■) 65°C, and the activity was assayed in 0.05 M imidazole buffer pH 6.0 at 65°C. (b) BXYL I was incubated without substrate at (○) 70 and (□) 75°C, and the activity was assayed in Mcllvaine buffer pH 5.0 at 75°C.

interesting, especially considering the range in which the enzymes present high activity and considerable stability, allowing their application in different industrial processes. The estimated half-lives of XYL I at 55, 60 and 65°C were 125, 16 and 6 min, respectively (Fig. 8a). BXYL I was highly stable at 60°C, retaining almost 100% of the activity after the 6 h of incubation (not shown), and the half-life values for this enzyme were 3 and 11 min at 70 and 75°C, respectively (Fig. 8b). Thermal stability is a desirable feature for many enzymes, once some bioprocesses can be unfeasible due to thermal inactivation of the enzymes. In fact, especially BXYL I was highly active and also very stable at high temperatures.

When incorporated to the enzymatic reaction, only the ions NH_4^+ and Na^+ (chloride or citrate) stimulated xylanase activity and also DTT at 10 mM and β -mercaptoethanol at both concentrations (Table 2) what can be justified by the avoidance of the oxidation of sulfhydryl groups or by the reduction of disulfide bridges in the presence of these agents, reestablishing the native conformation of the enzyme, or of some particular area near or in the active site. In relation to the BXYL I, no activation was observed in the presence of

Table 2
Effect of different ions and substances on XYL I and BXYL I from *P. janczewskii*.

Enzyme	XYL I		BXYL I	
Substance	Relative activity (%)			
	2 mM	10 mM	2 mM	10 mM
Control	100	100	100	100
CuCl ₂	ND	ND	78.3 ± 3.1	88.9 ± 8.4
ZnSO ₄	84.6 ± 5.5	83.1 ± 9.2	90.2 ± 9.5	87.3 ± 10.8
MnSO ₄	63.3 ± 8.7	49.4 ± 4.0	83.2 ± 4.4	89.3 ± 11.1
BaCl ₂	90.4 ± 7.4	108.4 ± 4.6	96.3 ± 8.7	111.9 ± 9.6
CaCl ₂	116.2 ± 9.4	100.3 ± 4.4	78.7 ± 9.0	84.43 ± 6.6
NH ₄ Cl	116.9 ± 11.8	131.3 ± 3.7	71.7 ± 9.6	91.8 ± 9.6
NaCl	131.9 ± 10.1	115.7 ± 10.1	78.7 ± 8.2	85.25 ± 4.9
SDS	ND	ND	56.6 ± 1.7	26.64 ± 1.6
PMSF	124.9 ± 23.1	55.5 ± 6.9	88.5 ± 9.9	97.95 ± 9.5
MgSO ₄	95.9 ± 3.1	104.7 ± 10.4	83.6 ± 6.2	108.6 ± 10.2
Sodium citrate	120.9 ± 6.5	120.7 ± 6.4	74.2 ± 3.3	80.3 ± 3.3
DTT	97.5 ± 2.7	127.5 ± 2.0	56.6 ± 2.9	74.6 ± 10.9
CoCl ₂	115.4 ± 9.3	83.5 ± 8.2	88.1 ± 4.1	101.6 ± 8.8
HgCl ₂	85.6 ± 3.9	46.2 ± 3.4	ND	ND
Pb(CH ₃ COO) ₂	83.3 ± 3.1	26.7 ± 1.1	106.2 ± 6.2	107.8 ± 6.0
EDTA	119.9 ± 5.7	58.3 ± 2.7	65.2 ± 10.1	81.97 ± 9.9
β-mercaptoethanol	129.3 ± 4.1	138.5 ± 5.5	82.8 ± 4.1	75.0 ± 10.6

Xylanase activity was assayed in 0.05 M imidazole buffer pH 6.0 at 65°C; β-xylosidase activity was assayed in McIlvaine buffer pH 5.0 at 75°C. Data are averages of duplicates. ND: not detected. Enzyme activities: see Table 1.

any ion or substance. This enzyme showed lower activity in the presence of DDT and β-mercaptoethanol indicating that it should have disulfide bonds on its structure. Inhibition by Hg²⁺ seems to be a common characteristic of xylanases, demonstrating the existence of cysteine thiol groups near or in the active site of the enzyme [43]. Usually, xylanases are completely inhibited by this cation as verified for many *Penicillium* xylanases [28,44,45,46], except for xyl 2 from *Aspergillus ochraceus* [47]. However, XYL I from *P. janczewskii* was only partially inhibited while BXYL I was completely inhibited by this ion. Nevertheless, inhibition of β-xylosidases is not as common as inhibition of xylanases, being observed only for those from *P. wortmanni* [31] but not for that from *P. sclerotiorum* [36]. The ion Cu²⁺ completely inhibited XYL I and moderately inhibited BXYL I. The ion Pb²⁺ at 10 mM strongly inhibited XYL I, but had no effect on BXYL I. The ions Zn²⁺ and Mn²⁺ also presented inhibitory effect on XYL I but not on BXYL I. The inactivation of both enzymes verified with EDTA at 10 mM, suggests that they may request metallic ion

Table 3
Hydrolysis of different substrates by combined action of XYL I and BXYL I from *P. janczewskii*.

Xylan	Product (g L ⁻¹)		
	Xylose	Xylobiose	Xylotriase
Oat spelt	0.13	1.29	0.02
Birchwood	0.05	0.22	ND
Beechwood	0.01	0.49	0.01

Each substrate at 4% (w/v) initial concentration was incubated with a combination of purified xylanase (2.8 U) and β-xylosidase (0.01 U) to a 15 mL reaction volume, pH 5.0, under mild agitation at 30°C. Samples were taken after 24 h and reactions were stopped by boiling the samples for 10 min. Data are averages of duplicates. ND: not detected.

as cofactor. The inactivation by SDS indicated the importance of hydrophobic interaction for maintenance of the tridimensional structure of the enzymes.

Carbohydrate analysis revealed that XYL I present 78% of carbohydrates. This amount is higher than that observed for xylanases I and II from *P. capsulatum* with 58.5 and 33.4% of glycosylation, respectively [27], and similar to the 71% glycosylation of xylanase I from *Aspergillus versicolor* [48]. The high glycosylation amount may have retarded the enzyme run in the SDS-PAGE, justifying the difference observed in the MW of the enzyme. BXYL I presented lower carbohydrate content, 10.5%, that is very similar to that observed for β-xylosidase from *Scytalidium thermophilum* [49].

XYL I hydrolyzed exclusively xylans with no activity towards Avicel or CMC. Despite many fungal β-xylosidases are also active on pNPArA [38], BXYL I was specific with no activity against this substrate, neither against pNPGal and pNPGlu, presenting activity only towards oNPX, corresponding to 22% of that on pNPX.

Xylans from different sources, i.e., grasses, cereals, soft and hardwoods, differ in their composition [50]. Due to these highly heterogeneous composition and structure, diverse blends of enzymes may be necessary to obtain maximum degradation of the polymer [51], considering that different xylanases have distinctive activities against xylans with diverse structures [52]. In this sense, the role of XYL I and BXYL I in the hydrolysis of natural substrates such as oat spelts, birchwood and beechwood xylans was examined by HPLC. Xylotriase, xyloetraose and mainly larger xylooligosaccharides (XOS) were the hydrolysis products of these substrates by XYL I (not shown); thus, this enzyme may be classified as an endo type enzyme.

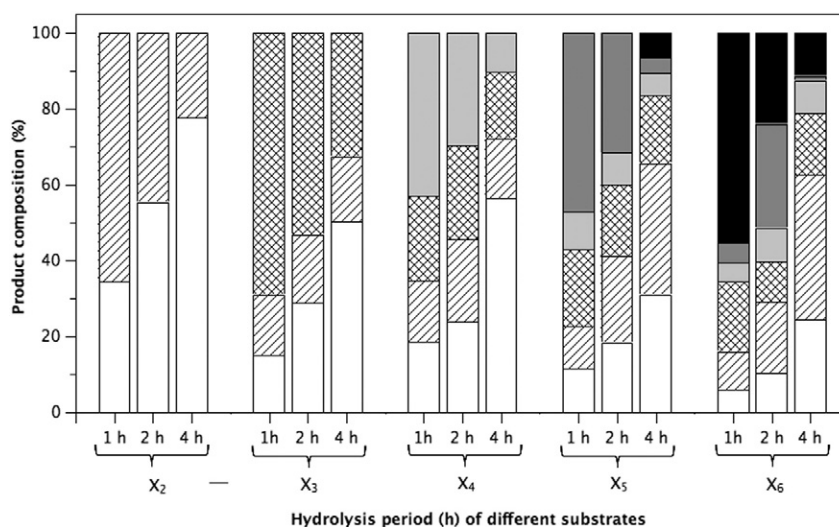


Fig. 9. Xylooligosaccharides hydrolysis by purified BXYL I from *P. janczewskii*. Reactions were carried out in pH 5.0 at 50°C. Data are averages of duplicates. Substrates: X₂ – xylobiose, X₃ – xylotriase, X₄ – xyloetraose, X₅ – xylopentaose, X₆ – xylohexaose. Products: (□) xylose, (▨) xylobiose, (▩) xylotriase, (▧) xyloetraose, (▦) xylopentaose, (■) xylohexaose.

Table 4
Kinetics of purified XYL I and BXYL I from *P. janczewskii*.

Substrate	Parameter			
	K_m (mg mL ⁻¹)	V_{max} (μ mol mL ⁻¹ min ⁻¹)	k_{cat} (s ⁻¹)	k_{cat}/K_m (mM ⁻¹ s ⁻¹)
<i>XYL I</i>				
Birchwood xylan	7.5	100.0	925.9	18.5
Oat spelts xylan	4.2	73.0	675.8	22.5
<i>BXYL I</i>				
pNPX	3.4	33.2	110.7	32.4

Xylanase activity was assayed in 0.05 M imidazole buffer pH 6.0 at 65°C; β -xylosidase activity was assayed in McIlvaine buffer pH 5.0 at 75°C. Data are averages of duplicates. pNPX – p-nitrophenyl β -D-xylopyranoside.

Besides, only a small amount of xylose could also be observed. XOS are high value-added products having importance as prebiotics in functional foods and also in pharmaceutical industry due to the plethora of beneficial effects for human health [53]. In this sense, the MW of xylan has inverse relation with the prebiotic effect, i.e., stimulation of *Bifidobacterium* and *Lactobacillus*, increases by decreasing the degree of polymerization [7].

BXYL I had no activity against polymeric xylans, as usually verified for most β -xylosidases. When purified xylooligosaccharides with different chain length were individually hydrolyzed by BXYL I (Fig. 9), the enzyme was able to hydrolyze up to xylohexaose, with xylose detected as initial product indicating an exo-type mode of action. The rate of xylose release decreased by increasing xylooligosaccharides chain length, and after 24 h-incubation, all substrates were converted to xylose. Besides, no transglycosylation (synthesis of oligosaccharides with higher degree of polymerization than the substrate) was detected even after 24 h-incubation. The activity of β -xylosidases can be related to the substrate polymerization degree; for example, those from *A. phoenicis* [40] and *A. versicolor* [54] hydrolyzed only up to xylotriase, while *S. thermophilum* β -xylosidase hydrolyzed up to xyloetraose [49].

The joint action of XYL I and BXYL I led to xylose release indicating that synergism in the degradation of these substrates and also accumulation of xylobiose was observed. Besides, great difference in degradation was observed among the different xylans (Table 3). Oat spelts xylan was more easily degraded releasing the highest xylose amount (0.13 g L⁻¹) and xylobiose (1.29 g L⁻¹) and xylotriase (0.02 g L⁻¹) were also detected. Birchwood xylan was hydrolyzed up to xylobiose (0.49 g L⁻¹) and the lowest xylose amount was obtained from this substrate (0.01 g L⁻¹). Intermediate amounts of xylose (0.05 g L⁻¹) and xylobiose (0.22 g L⁻¹) were observed from birchwood xylan. Synergistic effect in the degradation of substrates has also been verified with purified xylanases and β -xylosidases from *Paecylomyces thermophile* [55] and *Thermomonospora fusca* [56]. Oat spelts xylan is a more complex polymer containing 52.5% xylose, 22.3% arabinose, 15% glucose and 9.5% galactose [52]. Birchwood and beechwood xylans both are composed by >90% xylose according to the supplier and its accepted that xylan from hardwoods contain acetyl substituents [52]. These acetyl substituents may be playing a more important role than the structural complexity in hindering the polymers degradation by the XYL I and BXYL from *P. janczewskii* since oat spelts was more easily hydrolyzed than beechwood and birchwood xylans. Besides, beechwood xylan can be degraded up to xylobiose, with very low levels of xylose released.

The kinetics constants for XYL I were evaluated on birchwood and oat spelts xylans, and for BXYL I on pNPX (Table 4). Apparent K_m for XYL I was lower with oat spelts than with birchwood xylan indicating higher affinity of the enzyme for the former substrate. Similar K_m values are observed for xylanases from *Penicillium chrysogenum* [44] and *Penicillium* sp. 40 [57], although *P. chrysogenum* xylanase present almost the same values for both substrates. V_{max} values of XYL I from

P. janczewskii were higher for birchwood xylan that is a more soluble substrate, though k_{cat}/K_m relation is more appropriate to evaluate catalytic efficiency of an enzyme. Thus, despite higher k_{cat} values observed with birchwood xylan, the lower K_m values with oat spelts xylan indicated higher catalytic efficiency on the degradation of this substrate. The values K_m , V_{max} , k_{cat} and k_{cat}/K_m of BXYL I corresponded to 3.4 mM; 33.2 μ mol mL⁻¹ mg prot.⁻¹; 110.7 s⁻¹ and 32.4 mM⁻¹ s⁻¹, respectively. BXYL I was competitively inhibited by D-xylose, the main final product of xylan hydrolysis, presenting K_i of 6.0 mM using pNPX as substrate, similar to the value observed for the β -xylosidase from *A. versicolor* [54].

4. Conclusions

The established protocols proved to be adequate for the purification of the main extracellular xylanase and β -xylosidase produced by *P. janczewskii* since electrophoretic homogeneity was obtained at the end of the processes. Besides, it was demonstrated that at least two isoforms of each enzyme are produced by this fungal strain. The enzymes presented optimal activity in slightly acid pH and at temperatures above 50°C. Important properties such as synergism in the degradation of substrates and also prolonged stability at high temperatures were observed. Besides, the stability of the xylanase in the pH range from 6.0 to 10.0 indicates a potential application in processes that require these conditions, including textile treatment and in the pulp and paper industry. Studies on the molecular aspects of the enzymes and cloning in suitable expression vectors and a deeper study on the hydrolysis of different substrates, as well as their immobilization on carriers will be carried out in a future project.

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Conflict of interest

The authors declare that they have no conflict of interest.

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