





Immobilization and Stabilization of Beta-Xylosidases from *Penicillium janczewskii*

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Abstract β -Xylosidases are critical for complete degradation of xylan, the second main constituent of plant cell walls. A minor β -xylosidase (BXYL II) from *Penicillium janczewskii* was purified by ammonium sulfate precipitation (30% saturation) followed by DEAE-Sephadex chromatography in pH 6.5 and elution with KCl. The enzyme presented molecular weight (MW) of 301 kDa estimated by size exclusion chromatography. Optimal activity was observed in pH 3.0 and 70–75 °C, with higher stability in pH 3.0–4.5 and half-lives of 11, 5,

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and 2 min at 65, 70, and 75 °C, respectively. Inhibition was moderate with Pb^{+2} and citrate and total with Cu^{+2} , Hg^{+2} , and Co^{+2} . Partially purified BXYL II and BXYL I (the main β -xylosidase from this fungus) were individually immobilized and stabilized in glyoxyl agarose gels. At 65 °C, immobilized BXYL I and BXYL II presented half-lives of 4.9 and 23.1 h, respectively, therefore being 12.3-fold and 33-fold more stable than their unipuntual CNBr derivatives (reference mimicking soluble enzyme behaviors). During long-term incubation in pH 5.0 at 50 °C, BXYL I and BXYL II glyoxyl derivatives preserved 85 and 35% activity after 25 and 7 days, respectively. Immobilized BXYL I retained 70% activity after 10 reuse cycles of *p*-nitrophenyl- β -D-xylopyranoside hydrolysis.

Keywords β -Xylosidases · *Penicillium janczewskii* · Enzyme purification · Enzyme characterization · Enzyme immobilization · Enzyme stabilization

Abbreviations

<i>p</i> -NPX	<i>p</i> -Nitrophenyl β -D-xylopyranoside
PEG	Polyethylene glycol
PEI	Polyethylenimine
SDS	Sodium dodecyl sulfate

Introduction

The conversion of xylan into valuable products represents an important part of the effort to enable lignocellulose biomass processing in order to develop different ways in the manufacture of chemicals and renewable energy as well. Owing to its complex structure, the complete degradation of xylan demands numerous enzymes acting cooperatively. Among other enzymes from the xylanolytic complex, β -xylosidases (xylan 1,4-beta-xylosidase, EC 3.2.1.37) are exoglycosidases that hydrolyze non-reducing ends of xylooligosaccharides into xylose [1]. β -Xylosidases are critical for the complete degradation of xylans since they carry the greatest workload in relation to the number of cleaved glycosidic bonds, as well as in alleviating product inhibition of xylanases [2] and cellulases [3].

Interest in xylanolytic enzymes has increased in recent decades due to their applications in food, animal feed, pharmaceutical, and fuel and chemical industries. Notably, in processes in the pulp and paper and textile industries, cellulases must be absent in the enzymatic cocktail to preserve the cellulose fibers. A contemporary concern is the rational use of β -xylosidases in the complete hydrolysis of xylan to the final product, D-xylose, for their central role in many biotransformations [4–7].

Many fungal species are formidable sources of xylanolytic enzymes [8], and biomass degrading fungi rely on complex machineries that generally include both direct enzymatic depolymerization and also oxidative species [9]. After a new efficient producer is isolated, it is critical to purify and characterize the enzymatic constituents to comprehend their action towards substrates and their regulation and properties in order to develop more viable processes. Compared to free enzymes in solution, immobilized enzymes can be more robust and tolerant to environmental variations. Furthermore, the heterogeneity of immobilized systems permits easy recover of both biocatalyst and products, numerous re-use, continuous operation, rapid termination of reactions, and greater variety in bioreactor designs [10]. Immobilization of multimeric enzymes, such as many microbial β -xylosidases [11], can be a particular and complex problem in

which subunit dissociation can result in inactivation and even product contamination [12]. Currently, there are some reports dealing with immobilization and stabilization of microbial xylanases through multipoint covalent attachments [13–15], although, to the best of our knowledge, only one β -xylosidase has been immobilized by this technique, demonstrating the suitability of this support for immobilization of such important enzymes [16].

A *Penicillium janczewskii* strain isolated from the Atlantic Rainforest (São Paulo, Brazil) is a good producer of xylanolytic enzymes, but not of cellulases [17, 18]. The main extracellular β -xylosidase (BXYL I) produced in liquid cultures was purified and characterized as a 200 kDa dimeric enzyme with identical subunits, presenting optimum activity in pH 4.0 and at 75 °C [19]. In another study, the crude xylanase, β -xylosidase, and α -L-arabinofuranosidase were co-immobilized providing a biocatalyst active in the degradation of different arabinoxylans. In this preparation, the β -xylosidase was poorly stable under operational conditions [20]. In this work, we sought to purify and characterize a second extracellular β -xylosidase from *P. janczewskii* (BXYL II), which is present to a lesser extent in the culture filtrate. Besides, the immobilization of both β -xylosidases from this fungus was carried out aiming to obtain highly active and stable biocatalysts.

Materials and Methods

Materials

Agarose 4BCL was purchased from Agarose Bead Technologies (Madrid, Spain). *p*-Nitrophenyl- β -D-xylopyranoside, glycidol, potassium tetraborate tetrahydrate, sodium borohydride, sodium periodate, ethanolamine, *Leuconostoc* spp. dextran (molecular weight (MW) 15,000–25,000), polyethylenimine (PEI), and molecular weight standard proteins for electrophoresis and size exclusion chromatography were from Sigma-Aldrich Co. (St. Louis, MO, USA). Polyethylene glycol (PEG) was from Merck (Hohenbrunn, Germany). Cyanogen bromide (CNBr) Sepharose 4B was from GE Healthcare (Uppsala, Sweden). All reagents were analytical grade.

Methods

Results are mean of at least three experiments. Experimental error was $\leq 5\%$.

Strain Maintenance and Enzyme Production

P. janczewskii (CRM 1348) is deposited at The Central of Microbial Resources, CMR-UNESP, São Paulo, Brazil. Maintenance and enzyme production were carried out as previously described [19]. After fungal growth, mycelium was removed by vacuum filtration, and the cleared supernatant obtained after centrifugation (10,000 \times g, 20 min, 4 °C) was used as an enzyme source.

Determination of Enzyme Activity and Protein

β -Xylosidase activity was assayed using 5 mM *p*-nitrophenyl- β -D-xylopyranoside (*p*NPX) buffered solution [21]. BXYL I was assayed in 0.05 M sodium acetate buffer pH 5.0 and BXYL II in 0.05 M glycine HCl buffer pH 3.0. At appropriate time intervals, aliquots were

taken and the reaction was stopped with a saturated solution of sodium tetraborate. The released *p*-nitrophenolate was determined by reading absorbance at 405 nm. Assays were performed in duplicate.

During enzyme purification, protein determination was performed by the Lowry method [22], using bovine serum albumin as standard.

Enzyme Purification

BXYL I was partially purified using ammonium sulfate precipitation and ion exchange chromatography, based on previous protocol [19]. Chromatography with CM Sephadex C-50 was carried out in batch mode under mild agitation. After 3-h incubation, the supernatant was removed and adsorbed proteins were eluted with 0.05 M sodium acetate buffer pH 5.7 for additional 2 h.

BXYL II was precipitated with ammonium sulfate to 35% saturation. After centrifugation (10,000×*g*, 20 min, 4 °C), the supernatant source of BXYL I was separated (see below) and the precipitate was suspended in 0.05 M imidazole buffer pH 6.5 and dialyzed against the same buffer (6 h, three changes, 4 °C). After dialysis, the sample was chromatographed on a DEAE-Sephadex A-50 column (1.4 × 17 cm) equilibrated in the same buffer, 1.2 ml/min flow rate and 3.5 ml collected fractions. After washing the column with the same buffer, bound proteins were eluted with 100 ml of 0–1.0 M ascending KCl gradient prepared in the same buffer. Fractions with high β-xylosidase activity were pooled.

Partially purified BXYL I was employed for immobilization experiments. BXYL II was characterized in its free form and then immobilized. Purification was performed at 4 °C, and at the end of each step, protein concentration and enzymatic activity in the resultant sample were determined. Prior to immobilization, samples were concentrated by overnight dialysis against a 20% (w/v) polyethylene glycol MW 35,000 solution.

Determination of Molecular Weight by Size Exclusion Chromatography

The MW of BXYL II was calculated based on elution volumes of proteins (*V_e*) and blue dextran (*V_o*) in a Sephadex G-200 column (2.5 × 64.0 cm). Proteins were eluted with 0.05 M ammonium acetate buffer pH 6.8 at 0.3 ml/min flow rate and 3.0 ml collected fractions. β-Amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome c (12.4 kDa) were used as standard.

Electrophoresis

SDS-PAGE of the BXYL II sample from size exclusion chromatography was performed in 8–18% (w/v) gradient gel, and protein bands were stained with 0.1% (w/v) Coomassie brilliant blue R-250 [23]. Bovine carbonic anhydrase (29 kDa), egg albumin (45 kDa), bovine serum albumin (66 kDa), rabbit phosphorylase b (97 kDa), *Escherichia coli* β-galactosidase (116 kDa), and myosin from rabbit muscle (200 kDa) were used as standard.

Preparation of Agarose-Based Supports and Dextran Oxidation

Glyoxyl and monoaminoethyl-*N*-aminoethyl (MANAE) supports were prepared using 4% cross-link agarose beads, according to Mateo et al. [24] and Fernandez-Lafuente et al. [25],

respectively. After activation, the gels were washed with distilled water, vacuum dried (with the pores of agarose gels filled with water), and stored at 4 °C. Fully oxidized *Leuconostoc* spp. dextran was prepared as previously described [26].

Enzyme Immobilization

Stability of BXYL I and BXYL II in pH 10 In order to establish the conditions for immobilization on glyoxyl supports, the use of low temperature and protecting agents was evaluated aiming to stabilize the enzymes in alkaline conditions. The enzymes were 2-fold diluted in 10 ml of 0.2 M sodium bicarbonate buffer pH 10.0 containing 30 or 50% (w/v) trehalose, glycerol, and polyethylene glycol MW 6000 or 35,000. Incubation was carried out at in an ice-cold bath and at 25 °C. At different intervals, aliquots were withdrawn and the activity was assayed as described above. The immobilization protocol was further designed according to the stability of the soluble enzyme in the different conditions.

Immobilization on Glyoxyl Agarose BXYL I was 2-fold diluted in 0.2 M sodium bicarbonate buffer pH 10.0 containing 50% (w/v) PEG 35,000, to 10 ml final volume, 4 °C. The solution was added to 1.0 g of glyoxyl agarose support, and the suspension was gently stirred. The first immobilized derivative was prepared by carrying out immobilization for 2 h in an ice-cold bath. A second derivative was prepared by carrying out incubation initially in an ice-cold bath for 30 min. Then, the system was removed from cold to let the temperature gradually increase up to room temperature for an additional 40 min.

BXYL II immobilization was similar to described BXYL I; however, the pH was adjusted to 9.3–9.5 and contained 30% (w/v) PEG 35,000. After ice-cold incubation, the temperature was allowed to increase for an additional 1 h. A second derivative was prepared by carrying out overnight incubation (17 h) at room temperature.

After immobilization, the derivatives were reduced with 10 mg of sodium borohydride for 30 min and then washed abundantly with water and vacuum filtered.

Immobilization on MANAE Agarose and Post-Immobilization Techniques BXYL II was 2-fold diluted in 0.05 M sodium phosphate buffer pH 7.0, to a 20 ml final volume, 25 °C. The diluted enzyme solution was added to 2.0 g of MANAE-agarose support, and the suspension gently stirred for 30 min at 25 °C. After this period, the derivative was washed abundantly with the same buffer and vacuum filtered.

Dextran-coated derivative: 0.5 g of MANAE-agarose derivative, was added to 5 ml of fully oxidized dextran (as described above), pH adjusted to 7.0, and the suspension gently agitated at 25 °C. Incubation periods of 2 h and overnight were evaluated. Then, 5 ml of 0.1 M sodium bicarbonate buffer pH 10.0 was added, and the derivatives were reduced with 5 mg of sodium borohydride for 30 min.

PEI-coated derivative: 0.5 g of MANAE-agarose derivative was added to 5 ml of 50 g/l PEI MW 25,000 solution prepared in 0.025 M sodium phosphate buffer pH 7.0, and the suspension gently agitated at 25 °C. Incubation periods of 2 h and overnight were evaluated.

Glutaraldehyde-coated derivative: 0.5 g of MANAE-agarose derivative was added to 5 ml of 5 g/l glutaraldehyde solution, prepared in 0.025 M sodium phosphate buffer pH 7.0, and the suspension gently agitated at 25 °C. This treatment fully modifies the primary amino groups of the enzyme and the support with just one glutaraldehyde molecule [27, 28]. After 1 h, the suspension was filtered and washed with the same buffer to remove the excess of

glutaraldehyde. Additional buffered incubations for 2 h and overnight at 25 °C were evaluated in order to possibly achieve more intense enzyme-support cross-linking.

The derivatives were washed abundantly with water, filtered under vacuum, and stored at 4 °C.

Immobilization on CNBr-Activated Sepharose BXYL I and BXYL II were 4-fold diluted in 0.1 M sodium phosphate buffer pH 7.0 and immobilized under mild agitation, 15 min, 4 °C. The remaining reactive groups were blocked with ethanolamine, according to the supplier.

Immobilization Parameters Immobilization yield was defined as the ratio between activities in the final supernatant and in the blank of the soluble enzymes. Expressed activity was the ratio activity in the derivative suspension after immobilization and initial activity.

Free Enzyme and Derivative Characterization

Determination of Optima pH and Temperature Optimum pH for the free BXYL II was determined by performing enzyme assays in the pH range 3.0–8.0 using 0.05 M glycine HCl buffer pH 2.0, 2.5, and 3.0 and McIlvaine buffer pH 3.0–6.0, at 75 °C. Optimum temperature was determined by assaying activity in the 55–85 °C range, using 0.05 M glycine HCl buffer pH 3.0.

Enzymatic reactions with immobilized BXYL I and BXYL II on glyoxyl supports were carried out in 0.05 M glycine HCl buffer pH 2.0 and 3.0, 0.05 M sodium acetate buffer pH 4.2 and 5.0, and sodium phosphate buffer pH 6.0 and 7.0, at 75 °C. Optimum temperature was determined by assaying activity in the range from 60 to 85 °C, in 0.05 M sodium acetate buffer pH 5.0 for BXYL I and 0.05 M glycine HCl buffer pH 3.0 for BXYL II.

pH Stability Stability of free BXYL II at different pH was determined by incubating enzyme samples 1:2 (v/v) diluted in different buffers to compose a pH range of 2.0–6.0, using 0.05 glycine HCl buffer pH 2.0–3.0 and McIlvaine buffer pH 3.0–6.0. Incubation was carried out for 24 h at 4 °C. Enzyme activity was assayed in 0.05 M glycine HCl buffer pH 3.0 at 75 °C.

One gram of BXYL I and BXYL II glyoxyl derivatives was suspended in 10 ml of different buffers to compose the pH range 4.0–9.0. The following 0.05 M buffer systems were utilized: sodium acetate pH 4.2 and 5.0, sodium phosphate pH 6.0 and 7.0, and Tris HCl pH 8.0 and 9.0. The suspension was incubated at 50 °C, and at several time points, samples were withdrawn and the activity was assayed. The remaining activity was calculated as the ratio between activity at a given time and the activity at the beginning of incubation.

Thermal Stability The free BXYL II was incubated without substrate at 65, 70, and 75 °C. Aliquots were withdrawn at various time intervals for subsequent determination of residual activity. One gram of BXYL I and BXYL II glyoxyl derivatives was suspended in 10 ml of different buffers at different pH. Suspensions were incubated at different temperatures, and aliquots were withdrawn at various time intervals for subsequent determination of residual activity under the conditions stated above. The half-lives were calculated by the ratio of $\ln 2/K_d$. The inactivation constant (K_d) was determined by the equation: $\ln A = \ln A_0 - K_d \times t$, where A_0 and A are, respectively, the initial activity and the activity after time t (h).

Effect of Ions and SDS The influence of CuCl_2 , ZnSO_4 , MnSO_4 , BaCl_2 , CaCl_2 , NH_4Cl , NaCl , HgCl_2 , MgSO_4 , CoCl_2 , sodium citrate, lead acetate, and SDS on the activity of the free BXYL II was evaluated by adding these substances to the enzymatic reactions at 2 and 10 mM final concentrations. The results were expressed in relation to the control (no substance).

Catalyst Reuse Successive hydrolysis cycles of 3 mM *p*NPX prepared in 0.05 M sodium acetate buffer pH 5.0 were performed using BXYL I glyoxyl derivative at 1:10 proportion (w/v). Each cycle was carried out for 1 h at 50 °C under magnetic stirring (300 rpm). At the end of each cycle, the derivative was vacuum filtered, washed with 0.05 M sodium acetate buffer pH 5.0 and 1:10 (w/v) re-suspended in the same buffer to measure residual activity (activity before the first cycle regarded as 100%). It was then vacuum filtered again, and new substrate was added for the following cycle.

Results and Discussion

Purification of the Minor β -Xylosidase from *P. janczewskii*

A previous study purified and characterized the main β -xylosidase from this fungus (BXYL I) revealing the presence of a second β -xylosidase present in minor proportion in the crude extracellular extract [19]. The minor β -xylosidase from *P. janczewskii* (BXYL II) precipitated at 35% ammonium sulfate saturation, corresponding to 30% of the total activity. In the second purification step, the enzyme was submitted to anion exchange column chromatography in pH 6.5. Desorption of bound proteins was carried out with an ascending salt gradient. KCl was used since the enzyme was partially inactivated at high NaCl concentration. Most of the enzyme eluted with 0.65 M KCl, while most of non-target proteins eluted at higher KCl concentration (not shown).

Table 1 summarizes the purification of BXYL II from *P. janczewskii*. Precipitation with ammonium sulfate rendered low purification, although it was used as the first step in the process due to its high yield and especially for allowing the separation, in distinct samples, of the two β -xylosidases produced by this fungus. The enzyme was 3-fold purified, and the process presented 5.0% yield.

Characterization of the Minor β -Xylosidase from *P. janczewskii*

The sample from ion exchange chromatography was loaded into a size exclusion column eluting in a single sharp protein peak (not shown) with estimated native MW of 301 kDa, while some bands were observed on SDS-PAGE (Fig. 1), that may be attributed to contaminants eluting together with the enzyme due to its high MW. Probably, BXYL II is homotrimeric, with three 67 kDa subunits, as verified by the presence of a more intense band. The MW of BXYL II is similar to many fungal β -xylosidases which usually present MW above 100 kDa [29–33] but present MW higher than that of the BXYL I, whose native MW is 200 kDa [19].

BXYL II was highly active in acid pH, with optimal activity in pH 2.5–3.0. Intermediate activity values were observed in the pH range 3.5–5.0. In pH 2.0 or above pH 5.0, the activity decreased to lower levels. The enzyme presented high stability in the pH

Table 1 Purification of BXYL II from *P. janczewskii*

Step	Total activity (U)	Protein (mg)	Specific activity (U/mg prot.)	Yield (%)	Purification (fold)
Crude filtrate	12.1	91.8	0.1	100	1.0
Precipitate of 35% ammonium sulfate saturation	3.8	67.4	0.1	31.4	0.5
DEAE-Sephadex A-50 pH 6.5	0.6	1.9	0.3	5.0	3.0

β -Xylosidase activity was assayed in 0.05 M glycine HCl buffer pH 3.0 at 75 °C

range 3.0–4.5, in which 80% of activity was retained. Intermediate activity values were observed in pH 2.0, 2.5, 5.0, and 5.5, and in pH 6.0, only 30% activity was verified (Fig. 2a). The wide pH range in which the enzyme was stable, especially considering the 3.0–4.5 range, in which the enzyme presented high activity and stability, allows its application for prolonged periods in different process.

High activity was verified between 70 and 80 °C, with the optimum at 70–75 °C. Below 70 or above 85 °C, the activity decreased to less than 60% of the maximum (Fig. 2b). BXYL II presented optimal activity in slightly more acidic pH than BXYL I, that presents optimum activity in pH 5.0 [19]. Both β -xylosidases showed optimal activity at the same temperature, which is quite high, considering that they were produced by a mesophilic microorganism. Optimum activity in acid pH is a common feature of most fungal β -xylosidases [11]. Nevertheless, optimum activity at such high temperatures has not been reported for β -xylosidases from other *Penicillium* species, only for β -xylosidases from thermotolerant fungi such as *Aspergillus phoenicis* [34], *Aspergillus japonicus* [30], and *Aspergillus fumigatus* [35]. Activity at high temperatures is important, especially for application in processes requiring high temperatures in the pulp and paper industry and in baking, among others.

The estimated half-lives of BXYL II were 11, 5, and 2 min at 65, 70, and 75 °C, respectively (Fig. 3). BXYL I from *P. janczewskii* is slightly more stable than BXYL II presenting half-lives of 11 and 3 min at 70 and 75 °C, respectively [19].

Most of the evaluated ions presented no effect on the BXYL II from *P. janczewskii*. None of them activated the enzyme, whereas inhibition, at different levels, occurred in

Fig. 1 SDS-PAGE of BXYL II from *P. janczewskii*. Sample eluted from DEAE-Sephadex ion exchange chromatography (lane 1); molecular weight protein standards were bovine carbonic anhydrase (29 kDa), egg albumin (45 kDa), bovine albumin (66 kDa), rabbit phosphorylase b (97 kDa), *E. coli* β -galactosidase (116 kDa), and rabbit muscle myosin (200 kDa) (lane 2)

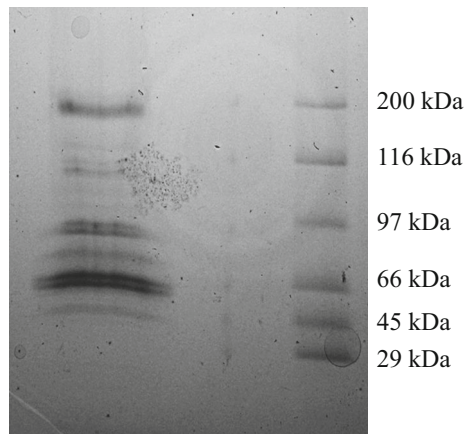
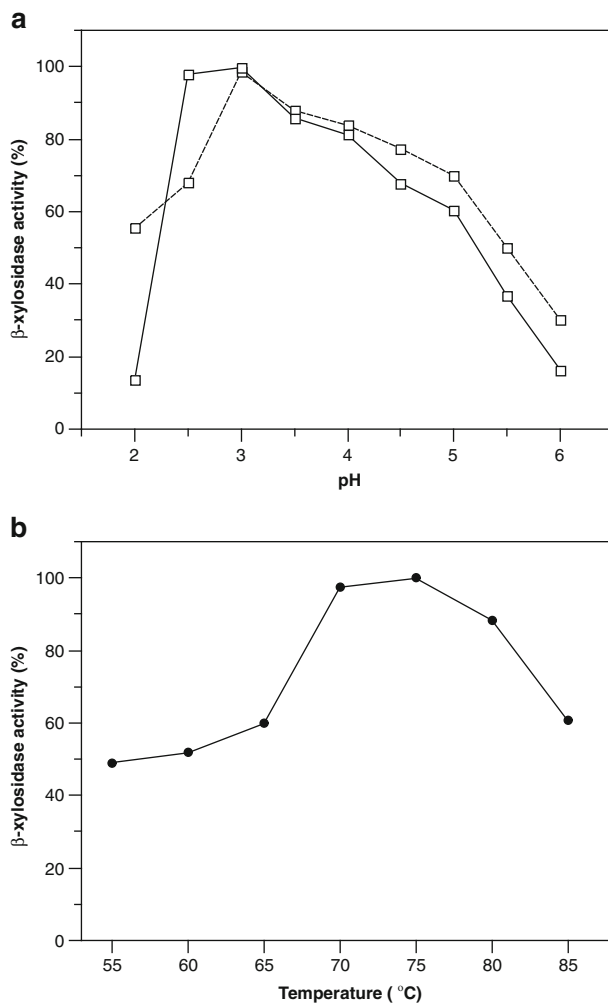
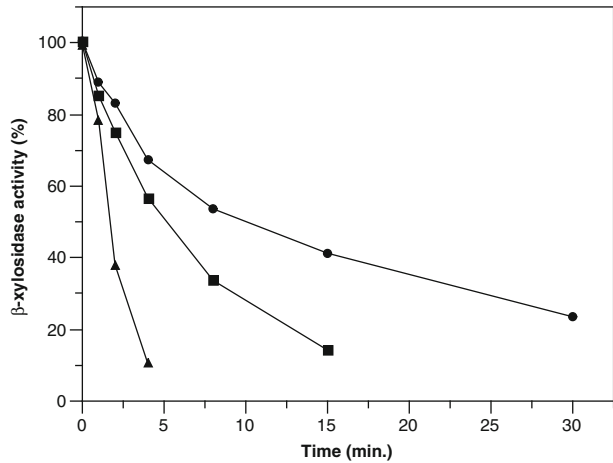


Fig. 2 Effect of pH (a) and temperature (b) on the free BXYL II from *P. janczewskii*. **a** Optimum activity (full lines) was determined by assaying enzyme activity in 0.05 M glycine HCl buffer pH 2.0–3.0 and McIlvaine buffer pH 3.0 to 6.0 at 75 °C. Stability (dashed line) was evaluated by 1:2 (v/v) diluting the enzyme in 0.05 glycine HCl buffer pH 2.0–3.0 and McIlvaine buffer pH 3.0–6.0. Incubation was carried out for 24 h at 4 °C, and then, activity was assayed in 0.05 M glycine HCl buffer pH 3.0 at 75 °C. **b** Enzyme activity was assayed in 0.05 M glycine HCl buffer pH 3.0. β -Xylosidase activity (%) (squares)



some cases: ZnSO_4 and $\text{Pb}(\text{CH}_3\text{COO})_2$ at 2 or 10 mM weakly inhibited (≈ 10 – 15% inhibition) the enzyme; CuCl_2 presented moderate effect at 2 mM ($\approx 25\%$ inhibition) and at 10 mM completely inhibited the enzyme. At 10 mM, sodium citrate moderately inhibited the enzyme ($\approx 35\%$), whereas CoCl_2 and HgCl_2 were strong inhibitors, completely depleting its activity. Moderate inhibition by ZnSO_4 , CoCl_2 , and sodium citrate is also observed for BXYL I, but lead acetate has no effect on this enzyme. Inhibition by CuCl_2 and HgCl_2 also occurs with BXYL I, although it is more susceptible to HgCl_2 , being completely inhibited at 2 mM, and more resistant to CuCl_2 , being only partially inhibited at 10 mM [19]. Inhibition by Hg^{2+} is not a general rule for β -xylosidases, and this effect was observed for the four purified β -xylosidases from *P. wortmanni* [36] and that from *Aspergillus nidulans* [37] but not for that from *P. sclerotiorum* [31]. BXYL II lost 25% activity with 2 mM SDS, and it was completely denatured at 10 mM. Activity loss in the presence of detergent indicates the importance of hydrophobic interactions to maintain the three-dimensional structure of the enzyme.

Fig. 3 Thermal inactivation course of free BXYL II from *P. janczewskii*. Enzyme activity was assayed in 0.05 M glycine HCl buffer pH 3.0 at 75 °C. β -Xylosidase activity (%) after incubation at 65 (circles), 70 (squares), and 75 °C (triangles)



Enzyme Immobilization

Different catalysts of BXYL I and BXYL II from *P. janczewskii* were prepared by using differently activated agarose supports and variable immobilization strategies, i.e., irreversible covalent immobilization on glyoxyl agarose and also reversible anionic immobilization on MANAE agarose (that can further become irreversible in the case of glutaraldehyde cross-linking). The thermal stabilities of these derivatives could be compared to each other and to those of the free enzyme and of the CNBr agarose derivative. CNBr derivatives were the controls, since they were prepared under very mild conditions, not allowing multipoint immobilization, but only by very reactive N-terminal. As a result, this derivative is reasonably similar to the free enzyme in its physico-chemical properties; however, to be bonded in a porous support, it has the advantage of not being susceptible to aggregation, hydrophobic interaction, etc., as opposition to the soluble protein [38].

Immobilization of BXYL I from *P. janczewskii*

Due to the low stability at pH 10.0, which is a required condition to immobilize on glyoxyl-activated supports, the use of low temperature and different substances was evaluated as protecting agents, stabilizing enzyme structure in this condition. When incubation was performed in an ice-cold bath, trehalose had no protective effect and glycerol presented an intermediate effect, sustaining 50% activity after 3-h incubation. Nevertheless, under room temperature, glycerol had no stabilizing effect, resulting in rapid depletion of enzyme activity (not shown). PEG was more efficient in stabilizing BXYL I. During ice-cold incubation, the enzyme slowly lost activity with almost no difference in stability verified among PEG of low or high MW. During incubation at room temperature, PEG MW 35,000 had a slightly more pronounced protective effect than PEG MW 6000 (S. 1).

Considering the high enzyme stability with PEG 35,000, in a first trial, immobilization was fully performed in an ice-cold bath (not shown). Nevertheless, the thermostability of this derivative was low (as presented below). Then, a second derivative was

prepared by carrying out immobilization initially with incubation in an ice-cold bath for 0.5 h; the suspension was subsequently removed from the cold, allowing the temperature to gradually increase to room temperature for an additional 1 h (S. 2). Thus, the final protocol involved two steps, the first at low temperature in which most of the enzyme became attached to the support and then a second with gradual temperature increase. The process presented 100% yield and the derivative 78% expressed activity. BXYL I immobilization on CNBr Sepharose presented 67% yield and the derivative 60% expressed activity.

Immobilization of BXYL II from *P. janczewskii*

Ionic immobilization of BXYL II on MANAE agarose rendered 92% yield in relation to the enzymatic activity. Immobilization activated the immobilized enzyme, and the expressed activity increased to 133%. In an attempt to fully stabilize the enzyme structure, this derivative was cross-linked with glutaraldehyde, polyethylenimine, and aldehyde-dextran at different conditions, which, at different levels, negatively influenced enzyme activity (Table 2).

Similarly to what was observed with BXYL I, the BXYL II also presented low stability in pH 10.0. Thus, immobilization of BXYL II on glyoxyl support followed a similar protocol to that established for BXYL. Immobilization, however, was carried out in pH 9.3–9.5, and the buffer contained a lower PEG concentration in order to avoid protein precipitation. Two glyoxyl derivatives were prepared, i.e., after the first stage of ice-cold incubation, immobilization was carried out for an additional 1.5 or 18 h, considering that long-term incubation could increase enzyme-support attachment, possibly rendering higher stability to the immobilized enzyme. After 0.5 h, no activity was detected in the supernatant and the derivatives with immobilization carried out for 1.5 or 17-h incubation presented 78 and 60% expressed activity, respectively. Immobilization of BXYL II on CNBr Sepharose resulted in 73.5% yield and the derivative expressed 63% activity.

Table 2 Post-immobilization strategies evaluated on MANAE derivative of BXYL II from *P. janczewskii*

Strategy		Expressed activity (%)	Half-life (h)
Coating/cross-linking substance	Incubation condition		
None	—	133	1.0
	1 h, pH 7, and 25 °C	38	NE
Glutaraldehyde (5 g/l)	Additional 2 h, pH 7, 25 °C	57	0.8
	Additional overnight incubation, pH 7, 25 °C	57	NE
Polyethylenimine MW 25,000 (50 g/l)	2 h, pH 7, 25 °C	34	0.8
	Additional overnight incubation, pH 7, 25 °C	10	NE
Dextran MW 15,000–25,000 (fully oxidized)	2 h, pH 7, 25 °C	41	0.5
	Additional overnight incubation, pH 7, 25 °C	10	NE

β -Xylosidase activity was assayed in 0.05 M Tris HCl buffer pH 3.0 at 25 °C

NE not evaluated

Characterization of BXYL I and BXYL II Derivatives

After establishment of the immobilization protocols, glyoxyl and CNBr derivatives were characterized in relation to thermal and pH stability and optimal pH and temperature.

Thermostability and Stability to pH

Stability at high temperature and in different pH is a very important aspect in enzymatic processes since the use of enzymes in industrial processes can be hindered by inactivation problems. Furthermore, the use of high temperature can contribute to avoid microbial contamination during the process.

When incubated at different temperatures and in pH 5.0 (Fig. 4), the BXYL I glyoxyl derivative (immobilization carried out initially in an ice-cold bath followed by temperature increase) was very stable up to 60 °C. At 70 °C, after 4 h of incubation, approximately 60% activity was retained, with half-life between 8 and 24 h. At 50 °C, this derivative still exhibited more than 75% activity even after 30-day incubation, while at 80 °C, all the activity was totally depleted after 30 min (not shown). On the other hand, the thermostability of the derivative for which immobilization was performed only in an ice-cold bath was much lower (half-life lower than 1 h at 70 °C) than that of the derivative obtained by following the two-stage protocol. This result clearly demonstrates that a higher temperature is necessary to increase the covalent bonds between the protein and the activated groups of the support, stabilizing its structure and providing much more thermostability to the immobilized enzyme.

The stability of the BXYL I glyoxyl derivative was evaluated in the pH range 4.0–9.0 at 50 °C (Fig. 5). The enzyme was stabilized in all pH, presenting residual activity above 60% even after 24-h incubation. In pH 9.0, the enzyme was somehow more stable, retaining about 90% activity during the entire assay period.

BXYL II glyoxyl derivatives were also incubated under different conditions, presenting high stability up to 60 °C and also in wide pH range, similarly to that verified for the BXYL I. During long-term incubation in pH 5.0 and 50 °C, this derivative retained 35% of the activity after 7 days (not shown).

Fig. 4 Thermal inactivation of immobilized BXYL I from *P. janczewskii*. Immobilization on glyoxyl support was carried out initially at low temperature in an ice-cold bath followed by temperature increase (*full lines*) and only at low temperature (*dashed lines*). Incubation was carried out in 0.05 M sodium acetate buffer pH 5.0, and the activity was performed in same buffer at 25 °C. β -Xylosidase activity (%) of glyoxyl derivatives incubated at 50 (*circles*), 60 (*squares*), and 70 °C (*diamonds*)

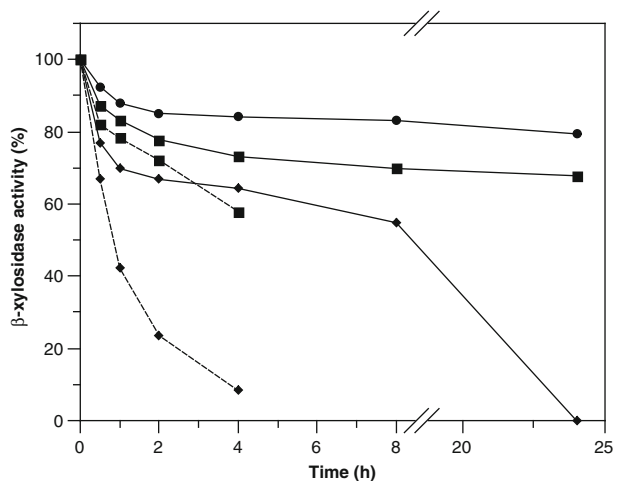
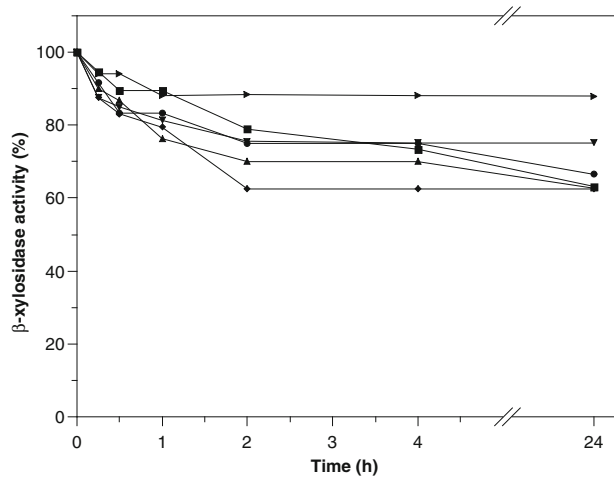


Fig. 5 Stability in different pH at 50 °C of immobilized BXYL I from *P. janczewskii*. BXYL I glyoxyl derivative was incubated in 0.05 M sodium acetate buffer pH 4.2 and 5.0, 0.05 M sodium phosphate buffer pH 6.0 and 7.0, and 0.05 M Tris HCl buffer pH 8.0 and 9.0. The activity was carried out in 0.05 M sodium acetate buffer pH 5.0. Residual β -xylosidase activity (%) after incubation in pH 4.2 (circles), 5.0 (squares), 6.0 (diamonds), 7.0 (triangles), 8.0 (inverted triangles), and (right-pointing triangles) 9.0

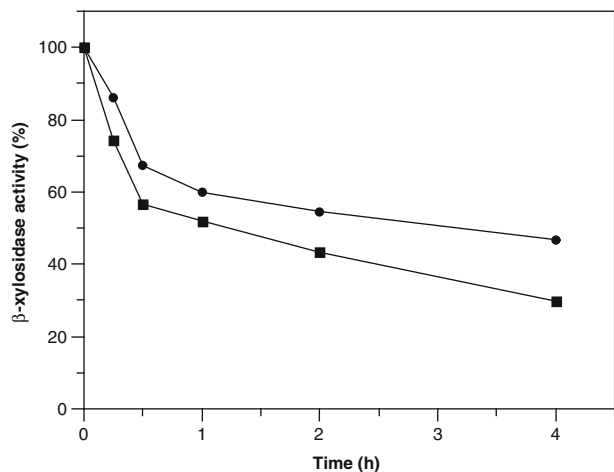


Comparing both BXYL II glyoxyl derivatives under incubation in pH 3.0 and at 65 °C, the glyoxyl derivative for which immobilization was carried out for 1.5 h reached higher stabilization. Long-term immobilization (17 h) did not increase enzyme stability. On the contrary, the stability was even reduced, i.e., the derivative prepared under 1.5-h incubation presented a half-life of 3.2 h, while that obtained after 17-h incubation presented a half-life of 1.3 h (Fig. 6).

MANAE derivatives of BXYL II incubated in pH 3.0 at 65 °C presented lower stabilities (Table 2). The half-life times of PEI-cross-linked and glutaraldehyde-cross-linked derivatives were both 0.8 h and that of the dextran-coated derivative was 0.5 h, lower than that of the uncoated MANAE derivative (1 h). In this sense, the glyoxyl derivative, for which immobilization was carried out for 1.5 h was considered the best BXYL II derivative and then further characterized.

When the stability of BXYL I and BXYL II glyoxyl derivatives was evaluated at high temperature and in different pH (Fig. 7), a similar inactivation pattern was observed for both immobilized enzymes, i.e., the highest stability was verified in pH 5.0, intermediate stability in pH 3.0, and the lowest stability in pH 7.0. Comparing both derivatives,

Fig. 6 Thermal inactivation of immobilized BXYL II from *P. janczewskii*. Immobilization on glyoxyl support was carried out initially at low temperature, and then, the temperature was allowed to increase during 1.5 and 17 h. Incubation was carried out in 0.05 M glycine HCl buffer pH 3.0 at 65 °C, and the activity was performed in same buffer at 25 °C. β -Xylosidase activity (%) of glyoxyl derivative prepared by 1.5 (circles) and 17-h incubation (squares)



immobilized BXYL II was more stable than BXYL I in all pH values. The half-lives at 65 °C in pH 5.0, 3.0, and 7.0 corresponded, respectively, to 4.9, 0.7, and 0.2 h for BXYL I and 23.1, 7.0, and 0.8 h for BXYL II.

CNBr derivatives incubated in pH 5.0 and 65 °C presented half-lives of 0.4 and 0.7 h, for BXYL I and BXYL II, respectively. BXYL I and BXYL II glyoxyl derivatives were therefore 12.3-fold and 33-fold stabilized in relation to their CNBr derivatives, respectively. Compared to the free enzyme, the BXYL II glyoxyl derivative was 128-fold thermostabilized. Furthermore, both BXYL I and BXYL II glyoxyl derivatives were more thermostable than the PEI-coated glyoxyl derivative of commercial β -xylosidase from *Selenomonas ruminantium* that presented half-life of 0.7 h at 55 °C [16].

Optima pH and Temperature

The activity profiles in different pH or temperatures showed that the immobilized enzymes retained their properties, i.e., activity in acid pH and at elevated temperature (Fig. 8). In

Fig. 7 pH stability of immobilized BXYL I (a) and BXYL II (b) from *P. janczewskii*. The derivatives were incubated in 0.05 M Gly HCl buffer pH 3.0, 0.05 M sodium acetate buffer pH 5.0, and 0.05 M sodium phosphate buffer pH 7.0 at 65 °C. β -Xylosidase activity (%) of glyoxyl derivatives incubated in pH 3.0 (circles), 5.0 (squares), and 7.0 (triangles) (full lines). Residual β -xylosidase activity (%) of CNBr derivatives incubated in pH 5.0 (inverted triangles) (dashed line)

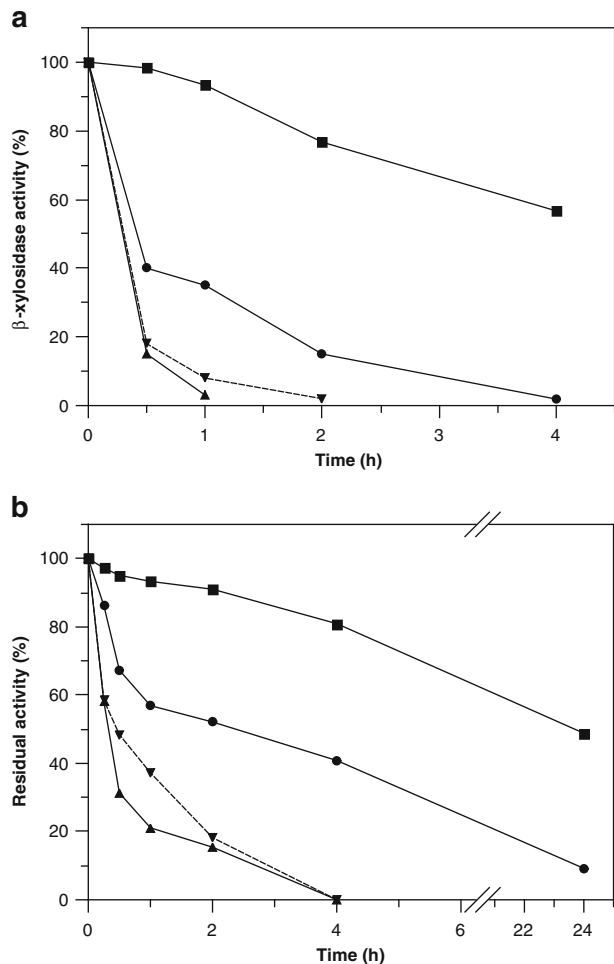
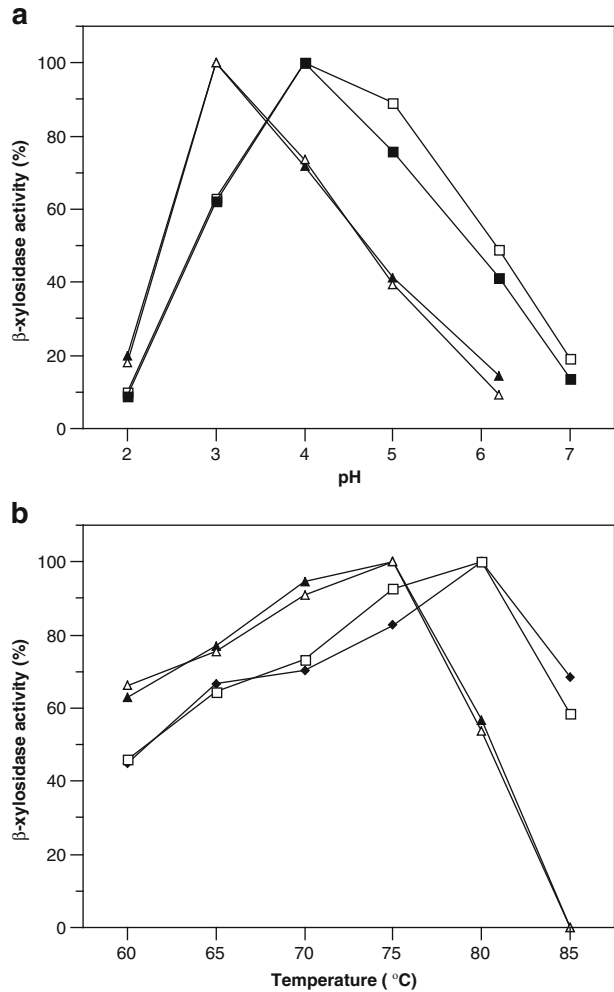


Fig. 8 Effect of pH (a) and temperature (b) on the activity of immobilized BXYL I and BXYL II from *P. janczewskii*. Enzyme activity was carried out in 0.05 M glycine HCl buffer pH 2.0 and 3.0, 0.05 M sodium acetate buffer pH 4.2 and 5.0, and sodium phosphate buffer pH 6.0 and 7.0 at 75 °C (a) and in 0.05 M sodium acetate buffer pH 5.0 for BXYL I and 0.05 M glycine HCl buffer pH 3.0 for BXYL II (b). β -Xylosidase activity (%) of derivatives BXYL I glyoxyl (filled squares), BXYL I CNBr (empty squares), BXYL II glyoxyl (filled triangles), and BXYL II CNBr (empty triangles)



relation to the free enzyme [19], immobilized BXYL I was active in a slightly more acid condition with optimum activity in pH 4.0 and also showed increase in optimum temperature from 75 to 80 °C. Immobilized BXYL II retained exactly the same properties of the free enzyme with optimal activity in pH 3.0 and at 70–75 °C.

Reuse Cycles

Considering that BXYL I is present in higher proportion in the crude extracellular extract and also its prolonged stability in pH 5.0 and at 50 °C in the immobilized form, the stability of the BXYL I glyoxyl derivative was evaluated during successive operational cycles of *p*NPX hydrolysis. The derivative retained 100% activity during five cycles, and after ten cycles, 70% of the activity was observed (not shown). This derivative was more stable than the glyoxyl derivative of *S. ruminantium* β -xylosidase, which presented 39% residual activity during eight *p*NPX hydrolysis cycles [16], that may be attributed to the less BXYL I subunits

(two subunits), while *S. ruminantium* β -xylosidase is tetrameric [39]. Moreover, due to its high operational stability, this derivative can be potentially used to complement the derivative containing the co-immobilized xylanase, β -xylosidase, and α -L-arabinofuranosidase from *P. janczewskii*, because the β -xylosidase is faintly stable in this biocatalyst [20].

Conclusions

The minor β -xylosidase from *P. janczewskii* shares some properties with the main β -xylosidase from this fungus, such as activity in moderately acid pH and at high temperatures. Despite their low stability in pH 10.0, both β -xylosidases can be stabilized at this condition and successfully immobilized by multipoint covalent immobilization on highly activated glyoxyl agarose gels. Specific protocols were designed for each enzyme since temperature and immobilization period revealed to be important parameters, which must be tightly regulated in order to obtain more stable derivatives. The immobilized enzymes were highly stabilized in wide pH range presenting much more stability than the free enzyme. Immobilized BXYL II is more stable at high temperatures than BXYL I, while immobilized BXYL I is much more stable under long-term incubation. The derivatives present promising characteristics, such as high stability, offering advantages over the free enzyme. Due to the long-term stability and operational stability, BXYL I glyoxyl derivative is proposed to be jointly used with a previously developed biocatalyst containing co-immobilized xylanase, β -xylosidase, and α -arabinofuranosidase activities for the complete degradation of arabinoxylans.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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