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Purification and properties of a thermostable extracellular β-D-xylosidase produced by a thermotolerant *Aspergillus phoenicis*

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A β-D-xylosidase was purified from cultures of a thermotolerant strain of *Aspergillus phoenicis* grown on xylan at 45°C. The enzyme was purified to homogeneity by chromatography on DEAE-cellulose and Sephadex G-100. The purified enzyme was a monomer of molecular mass 132 kDa by gel filtration and SDS-PAGE. Treatment with endoglycosidase H resulted in a protein with a molecular mass of 104 kDa. The enzyme was a glycoprotein with 43.5% carbohydrate content and exhibited a pI of 3.7. Optima of temperature and pH were 75°C and 4.0–4.5, respectively. The activity was stable at 60°C and had a K_m of 2.36 mM for *p*-nitrophenyl-β-D-xylopiranoside. The enzyme did not exhibit xylanase, cellulase, galactosidase or arabinosidase activities. The purified enzyme was active against natural substrates, such as xylobiose and xylotriose. *Journal of Industrial Microbiology & Biotechnology* (2001) **26,** 156–160.

Keywords: β-D-xylosidase; xylanase; *Aspergillus phoenicis*; thermostability

Introduction

Xylan is the main component of hemicellulose, which is present in large amounts in nature. It represents a potentially renewable energy resource that could be utilized to improve the technology of bioconversion of plant biomass into useful products. Xylan is a complex molecule consisting of a backbone of β-1,4-linked xylose residues. However, in nature, acetyl 4-*O*-methyl-D-glucuronosyl and/or L-arabinofuranosyl substitute some residues. Because of the complexity of the xylan molecule, its complete breakdown requires the concerted action of several enzymes, such as endoxylanase, β-D-xylosidase, α-L-arabinofuranosidase, α-glucuronidase, acetylxylan esterase and phenolic acid esterases. Xylanases have potential applications in food and feed industries, textile processes, enzymatic saccharification of lignocellulosic materials and waste treatment [9,14,22]. β-D-Xylosidase (β-D-xyloside xylohydrolase, EC 3.2.1.37) is an exoglycosidase that hydrolyzes short xylooligosaccharides and xylobiose from the nonreducing end to liberate xylose. The fermentation of xylose or xylose-containing hydrolyzates for ethanol production or bioconversion into xylitol has been reviewed recently [2,24].

The potential biotechnological applications of thermostable enzymes have stimulated a search for strains expressing the activities that possess desired features. In the course of such a quest, a thermotolerant *Aspergillus* strain, grown at 45°C, was isolated from Brazilian soil and bagasse sugar cane composting. Here, we report the purification and biochemical characterization of a thermostable β-D-xylosidase activity from *Aspergillus phoenicis*.

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Materials and methods

Microorganism and culture conditions

An *Aspergillus* strain (RP-02, from our collection) was isolated in our laboratory from Brazilian soil and bagasse sugar cane compost during a screening program for β-D-xylosidase and xylanase production. This isolate was classified as *A. phoenicis* [19]. The fungus was cultivated in liquid medium (SR) of the following composition (w/vol): 0.012% $MgSO_4$; 0.015% KH_2PO_4; 0.05% $NH_4H_2PO_4$; 0.02% peptone; 0.45% yeast extract and 1% xylan birchwood, pH 6.0. Growth and production of enzymes were followed in 250-ml Erlenmeyer flasks, each containing 50 ml of culture medium and incubated with agitation (100 rpm), at 45°C for 72 h.

Enzyme assays

β-D-Xylosidase was determined using a 3.68 mM *p*-nitrophenyl-β-D-xylopyranoside substrate in McIlvaine buffer [18], pH 4.0. After incubation at 75°C for varying times, the reaction was stopped by adding two volumes of a saturated solution of sodium tetraborate and the absorbance was measured at 405 nm. One unit of β-D-xylosidase activity was defined as the amount that liberates 1 μmol of *p*-nitrophenol per minute. Other aryl-β-glycosidase activities were assayed with the appropriate substrates, under the conditions described for β-D-xylosidase.

Purification of extracellular β-D-xylosidase

The crude filtrate (250 ml) was dialyzed overnight against 10 mM sodium phosphate buffer, pH 8.8 buffer (A) and applied to a DEAE-cellulose column (1.2×4.5 cm) equilibrated with the same buffer and 15 ml fractions were collected. β-D-Xylosidase was eluted with a linear gradient of sodium chloride (0–0.4 M) in

Table 1 Effect of carbon sources (sugars and industrial residues) on *β*-xylosidase production by *A. phoenicis*[a]

Carbon source	Concentration (%)	Protein (mg total)	Specific activity U (mg protein)$^{-1}$
None		1.5	8.6
Sugars			
Avicel	1	1.7	nd[b]
Cellobiose	2	1.8	95.4
Fructose	2	3.6	45.7
Glucose	2	4.7	34.5
Maltose	2	4.4	43.4
Raffinose	2	1.3	nd
Starch	1	1.6	82.9
Sucrose	2	1.9	nd
Xylan	1	2.3	278.3
Xylose	1	1.8	146.7
Industrial residues			
Bagasse sugar cane	1	2.9	219.9
Cassava flour	1	3.3	nd
Maize pith	1	4.0	112.3
Oatmeal	1	3.1	nd
Rice peal	1	2.7	nd
Wheat raw	1	5.2	35.0

[a]The microorganism was grown at 25°C, for 72 h, in 50 ml of SR medium, pH 6.0.
[b]Not detected.

buffer A. Active fractions were pooled, dialyzed against water and lyophilized. The protein sample was redissolved in 100 mM sodium acetate buffer, pH 5.5 and applied to a Sephadex G-100 column (55.5×1.3 cm). Fractions containing *β*-D-xylosidase activity were pooled, dialyzed against distilled water and used for biochemical characterization.

Analytical methods

Protein was estimated by the Lowry method [17] using bovine serum albumin as standard. Total neutral carbohydrate was estimated by the method of Dubois *et al* [7]. Polyacrylamide gel electrophoresis under nondenaturing conditions was performed by the Davis method [6] and under denaturing conditions (SDS-PAGE) according to Laemmli [16]. Protein was stained with silver as recommended by Blum *et al* [3]. *β*-D-Xylosidase activity was revealed by fluorescence on 6% PAGE using methylumbelliferyl-4,7-*β*-D-xyloside.

Table 2 Purification of the extracellular *β*-xylosidase activity from *A. phoenicis*

Step	Total protein (mg)	Total units (U)	Specific activity U (mg protein)$^{-1}$	Yield (%)	Purification (fold)
Crude enzyme	117.0	5440.0	46.5	100.0	1.0
DEAE-cellulose	7.1	1544.8	216.4	28.4	4.7
Lyophilization	4.7	662.7	140.1	12.2	3.0
Sephadex G-100	0.7	533.4	820.6	9.8	17.7

HPLC analysis

The hydrolysate of xylanase was treated with *β*-D-xylosidase, and the resulting products were determined by high performance liquid chromatography (HPLC) on a Lichrossorb RP-18 column (4 μm; 250×10 mm; Merck) equilibrated and eluted with water as the mobile phase at a flow rate of 1.0 ml min^{-1}. Peaks were detected by monitoring the refractive index and identified by comparing elution times to those of appropriate standards (xylose, xylobiose, xylotriose). Peak areas were estimated by Integrator Waters Data Module.

Results and discussion

Optimization of culture conditions for *β*-D-xylosidase production

Several sugars were used as carbon sources in the culture medium, but *β*-D-xylosidase was significantly produced only

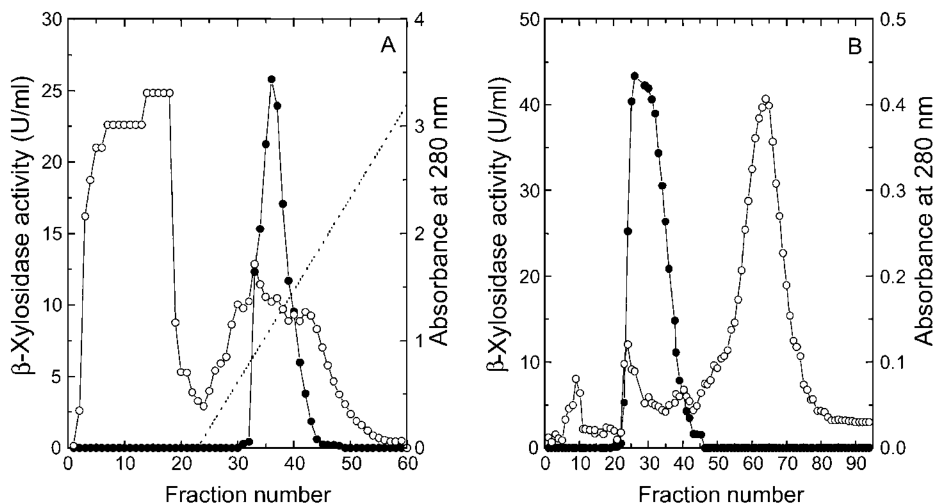

Figure 1 Elution profile of the *β*-xylosidase activity from DEAE-cellulose (A) and Sephadex G-100 (B) chromatography columns. Symbols: (○) absorbance 280 nm; (●) *β*-xylosidase activity; (...) linear gradient of sodium chloride (0–0.4 M). Details in Materials and methods section.

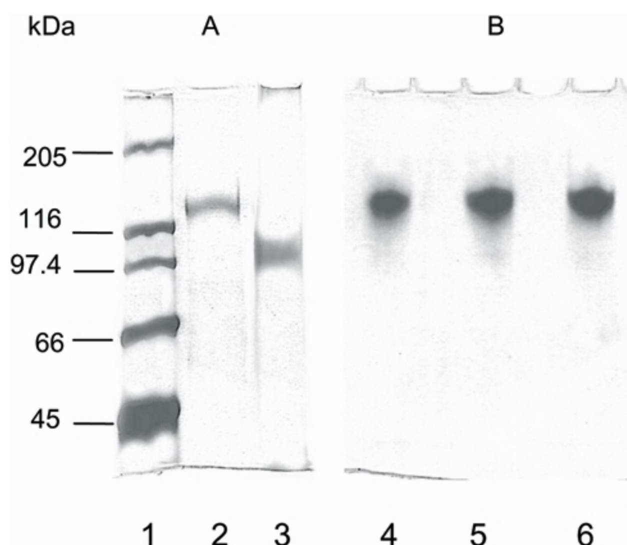

Figure 2 SDS-PAGE (A) and PAGE (B) of the purified β-xyloside. Line 1: molecular weight markers (myosin 205 kDa; β-galactosidase 116 kDa; phosphorylase *b* 97.4 kDa; bovine albumin 66 kDa; egg albumin 45 kDa and carbonic anhydrase 29 kDa). Lines 2 and 3: purified β-xylosidase (1 μg) and deglycosylated β-xylosidase (1 μg), respectively. Lines 4–6: represent 4, 6 and 8 μg of purified enzyme.

with xylan, xylose or with an industrial raw waste, bagasse sugar cane and maize pith (Table 1). Others, such as cassava flour, oatmeal, rice peel and wheat raw were poor inducers. Glucose, fructose, maltose, cellobiose and avicel repressed synthesis of this enzyme. Experiments with culture medium supplemented with 1% xylan (w/vol) and 1% (w/vol) glucose showed that β-D-xylosidase activity might be under the control of catabolic repression. Similar results were obtained with *Aspergillus sydowii* MG49 [8] and *Humicola grisea* var. *thermoidea* [1]. The highest levels of intracellular and extracellular β-D-xylosidase activities were verified at 72 h in cultures supplemented with xylan. However, when bagasse sugar cane was used as a carbon source the enzyme was delayed, and maximum enzyme secretion was attained only at 85 h.

Purification of β-D-xylosidase

β-D-Xylosidase was purified after two chromatographic steps as described in the methods. Figure 1A and B shows the elution profiles from DEAE-cellulose and Sephadex G-100 columns, respectively. The specific activity was 821.0±85.0 U (mg protein)$^{-1}$ (n=4) and a 17.7 fold purification was achieved (Table 2). The specific activity of *A. phoenicis* β-D-xylosidase was higher than others described in the literature [1,11]. The purified enzyme, when run under nondenaturing PAGE and SDS-PAGE, produced a single band (Figure 2). The activity band, revealed with methylumbelliferyl-4,7-β-D-xyloside was coincident with the protein band stained with silver.

Molecular properties

β-D-Xylosidase had a molecular mass of about 132 kDa by SDS-PAGE and by Bio Sil SEC-400 (Bio Rad), suggesting that the enzyme was a monomeric protein similar to other β-D-xylosidases [11,23]. Treatment with endoglycosidase H (Roche Mol. Biochem.) resulted in a protein with molecular mass of 104 kDa. Electrofocusing of the purified enzyme showed that its pI was about 3.7. This value is close to those previously reported for β-D-xylosidase purified from other fungi [1,21]. The carbohydrate content of the enzyme was estimated as 43.5±1.7% (n=3), using mannose as a standard. Other β-D-xylosidases described in the literature are also glycosylated [4,10].

Stability and effect of temperature and pHI on β-D-xylosidase activity

The temperature for maximum activity was 75°C, exhibiting a sharp peak at this temperature (Figure 3A). This value was

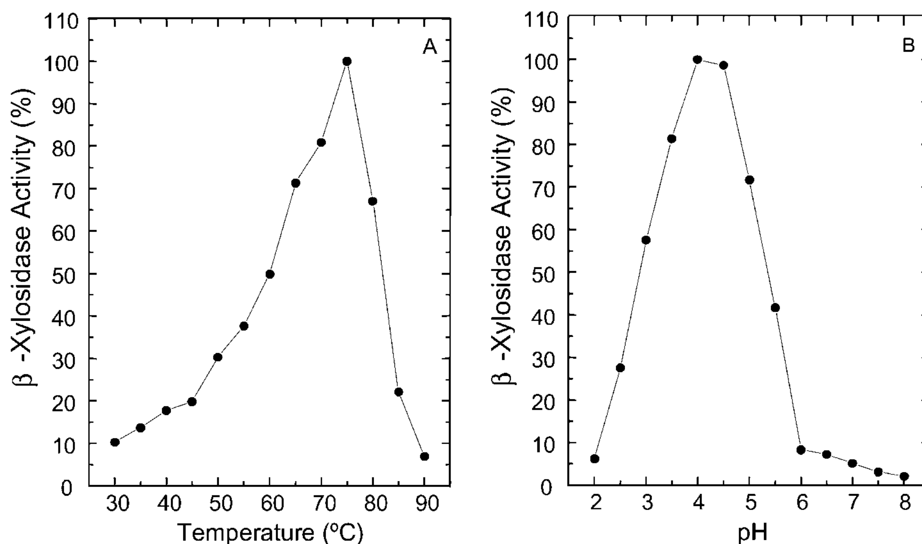

Figure 3 Effect of temperature (A) and pH (B) on purified β-xylosidase activity. The assays were performed with McIlvaine buffer pH 4.0 at 30–90°C for the first experiment and with McIlvaine buffer pH 2.0–8.0 at 75°C for the second one.

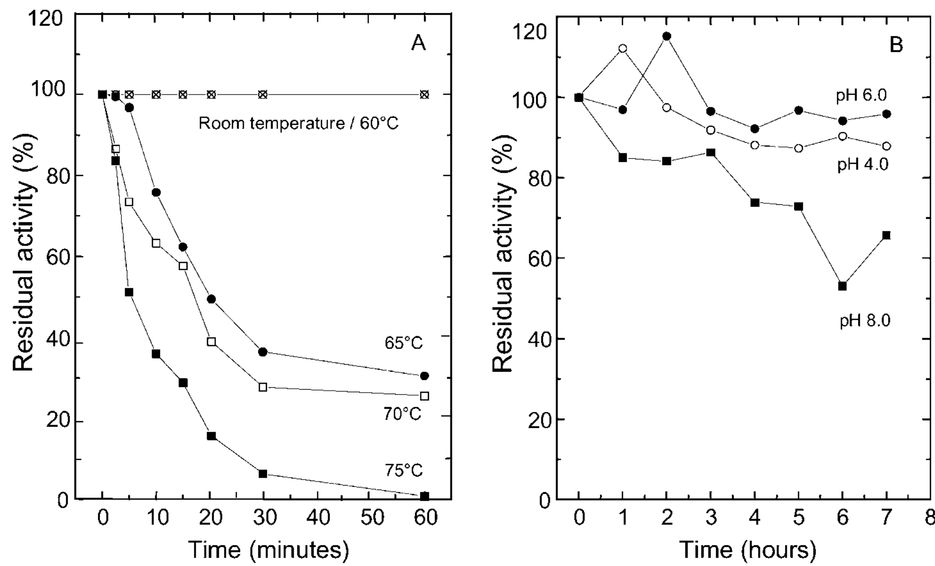

Figure 4 Stability of β-xylosidase activity at different temperature (A) and pH values (B). Stability of β-xylosidase was determined by incubating samples, at different times, at room temperature 25°C, (×), 60°C (○), 65°C (●), 70°C (□) or 75°C (■). Then the activity was determined as described in Materials and methods. pH stability was determined by preincubating with McIlvaine buffer at pH 4.0 (○), 6.0 (●) or 8.0 (■), before the β-xylosidase assay.

about 5–35°C above those reported for other *Aspergillus* strains, such as *A. nidulans* [15], *A. niger* [11,20,25], *A. oryzae* [13], *A. pulverulentus* [21] and *A. terreus* [5]. The optimum pH exhibited by *A. phoenicis* β-D-xylosidase activity was in the range of 4.0–4.5 using McIlvaine buffer (Figure 3B), which is close to those published for other β-D-xylosidases [5,13,20,25]. The purified enzyme was completely stable up to 4 h at 60°C, or 21 days at room temperature (25°C). Higher temperatures (65, 70, 75°C) significantly diminished the activity in 60 min (Figure 4A). The activity was stable for 7 h, at 4°C, in McIlvaine buffer pH 4.0 and 6.0 (Figure 4B). The properties cited above are different from those previously published for β-D-xylosidase activity from *A. phoenicis* [26], which presented a molecular mass of 95 kDa, optimum temperature and pH of 65°C and 3.5, respectively.

Table 3 Effect of metal ions and some compounds on purified β-xylosidase

Compounds[a]	Residual activity (%)
Control	100
$MgCl_2$	122.0
$CaCl_2$	110.4
$MnCl_2$	108.4
β-Mercaptoethanol	98.5
EDTA	72.8
NH_4Cl	69.7
NaCl	68.2
$ZnCl_2$	68.2
$BaCl_2$	60.9
$CuCl_2$	59.8
$HgCl_2$	9.2
p-Chloromercuribenzoate	Nd[b]

[a]Final concentration 1 mM.
[b]Not detected.

Effect of metal ions, β-mercaptoethanol and p-chloromercuribenzoate

β-D-Xylosidase activity was slightly stimulated by 1 mM Mg^{2+}, Ca^{2+} and Mn^{2+} (Table 3). Maximal activation was verified with 1.5 mM Mg^{2+} (33%). It was totally inhibited by *p*-chloromercur-

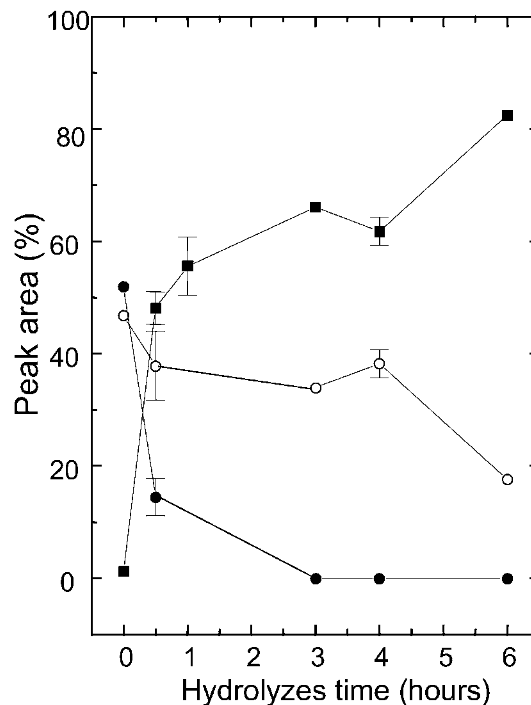

Figure 5 Kinetics of hydrolyses of xylooligosaccharide mixtures by β-D-xylosidase from *A. phoenicis*. Samples were removed at intervals up to 360 min and analyzed by HPLC as described in Materials and methods. Symbols: (■) xylose; (○) xylobiose; and (●) xylotriose.

ibenzoate and partially by Hg^{2+}, Cu^{2+}, Ba^{2+}, Zn^{2+}, Na^{+} and NH^{4+}. β-D-Mercaptoethanol did not significantly affect the activity. The effect of p-chloromercuribenzoate suggests the involvement of sulphydryl groups in the catalytic activity. Similar results have been published for the β-D-xylosidase of *A. niger* [11] and *A. sydowii* [8].

Substrate specificity and kinetic parameters

The oligosaccharides produced by the action of xylanase on xylan were analyzed by HPLC and showed xylobiose and xylotriose, each at the same percentage (Figure 5) as controls (0 h). We observed for the first four that the main product was xylose, the fragments generated by hydrolysis of one xylosyl linkage to xylotriose rather than of xylobiose. The purified enzyme hydrolyzed xylotriose more rapidly, and disappeared within 3 h. Xylobiose had no significant degradation in the same time. Similar results have been published for the β-D-xylosidase of *A. niger* [11]. The purified β-D-xylosidase from *A. phoenicis* did not exhibit xylanase, carboxymethylcellulase, avicelase, galactosidase, and arabinosidase activities. Traces of glucosidase activity were detected, similar to β-D-xylosidase from other fungi [12]. The enzyme exhibited typical Michaelis–Menten kinetics with p-nitrophenyl-β-D-xylopyranoside as the substrate. K_m and V_{max} values were 2.36 ± 0.54 mM and 920.75 ± 40.45 U (mg protein)$^{-1}$, respectively. These values are in the range of those reported for other β-D-xylosidases [1,4].

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