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ORIGINAL PAPER

Homologue expression of a β-xylosidase from native *Aspergillus niger*

A. Amaro-Reyes · B. E. García-Almendárez · D. G. Vázquez-Mandujano · S. Amaya-Llano · E. Castaño-Tostado · R. G. Guevara-González · O. Loera · C. Regalado

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Abstract Xylan constitutes the second most abundant source of renewable organic carbon on earth and is located in the cell walls of hardwood and softwood plants in the form of hemicellulose. Based on its availability, there is a growing interest in production of xylanolytic enzymes for industrial applications. β-1,4-xylan xylosidase (EC 3.2.1.37) hydrolyses from the nonreducing end of xylooligosaccharides arising from endo-1,4-β-xylanase activity. This work reports the partial characterization of a purified β-xylosidase from the native strain *Aspergillus niger* GS1 expressed by means of a fungal system. A gene encoding β-xylosidase, *xlnD*, was successfully cloned from a native *A. niger* GS1 strain. The recombinant enzyme was expressed in *A. niger* AB4.1 under control of *A. nidulans gpdA* promoter and *trpC* terminator. β-xylosidase was purified by affinity chromatography, with an apparent molecular weight of 90 kDa, and showed a maximum activity of 4,280 U mg protein^{-1} at 70°C, pH 3.6. Half-life was 74 min at 70°C, activation energy was 58.9 kJ mol^{-1}, and at 50°C optimum stability was shown at pH 4.0–5.0. β-xylosidase kept residual activity >83% in the presence of dithiothreitol (DTT), β-mercaptoethanol, sodium dodecyl sulfate (SDS), ethylenediaminetetraacetate (EDTA), and Zn^{2+}. Production of a hemicellulolytic free xylosidase showed some advantages in applications, such as animal feed, enzymatic synthesis, and the fruit-juice industry where the presence of certain compounds, high temperatures, and acid media is unavoidable in the juice-making process.

Keywords *Aspergillus niger* GS1 · β-xylosidase activity · Thermostability · Homologue expression

A. Amaro-Reyes · B. E. García-Almendárez · D. G. Vázquez-Mandujano · S. Amaya-Llano · E. Castaño-Tostado · C. Regalado (✉)
DIPA, PROPAC. Facultad de Química, Universidad Autónoma de Querétaro, C.U. Cerro de las Campanas s/n. Col. Las Campanas, 76010 Qro. Querétaro, Mexico
e-mail: carlosr@uaq.mx; regcarlos@gmail.com

R. G. Guevara-González
C.A Ingeniería de Biosistemas, Facultad de Ingeniería, Universidad Autónoma de Querétaro, C.U. Cerro de las Campanas s/n. Col. Las Campanas, 76010 Qro. Querétaro, Mexico

O. Loera
Dpto. de Biotecnologia, Universidad Autónoma Metropolitana-Iztapalapa, 09340 Mexico, DF, Mexico

Introduction

Xylan constitutes the second most abundant source of renewable organic carbon on earth and is located in the cell walls of hardwood and softwood plants in the form of hemicellulose [28]. Endo-β-1,4-xylanases (EC 3.2.1.8) and β-1,4-xylan xylosidase (EC 3.2.1.37) are key enzymes that hydrolyze xylan into xylooligosaccharides [4, 27]. β-xylosidase hydrolyses the terminal xylose unit from the nonreducing end of the xylooligosaccharides arising from endo-1,4-β-xylanase activity [22]. This is important, as β-xylosidase may relieve the end-product inhibition of endoxylanases and is also effective in transglycosylation reactions in which monosaccharide units or alcohols are attached to or cleaved from xylose units [9]. There is a growing interest in developing high-yield and low-cost production of xylanolytic enzymes for industrial applications, such as bioconversion of agroindustrial residues to biofuels, low-calorie sweeteners, and pharmacological products [20]. The worldwide market of these enzymes is

around 200 million US dollars per annum [14]. Therefore, the search for strains with the generally recognized as safe (GRAS) status that will grow in low-cost substrates that will optimize xylanolytic enzyme production represents an ultimate goal in this field of research. Filamentous fungi are more attractive than bacteria as potential producers of these enzymes because fungi secrete higher enzyme levels into the culture medium [16]. *Aspergillus niger* is a saprophytic fungus well known for its production and secretion of a variety of hydrolytic enzymes, contributing to its ability to degrade plant polysaccharides such as cellulose, hemicellulose, pectin, starch, and inulin [31]. Several studies have reported a variety of hemicellulolytic enzymes induced with various hemicellulosic residues (corn cob, sugar-cane bagasse, wheat bran, wheat straw, and rice straw) as the main carbon source using *Aspergillus* species [12, 18]. Nevertheless, some industrial applications require xylanases and xylosidases free of cellulase, among other hemicellulolytic activities, and enzymatic stability is also required over a broad range of temperatures and pH values [20].

A strain of *Aspergillus* was isolated from Mexican copra paste that produces a variety of cell-wall-degrading enzymes using different substrates upon solid-state fermentation [23]. Molecular identification of this novel and potentially useful *Aspergillus* strain and its xylanolytic genes have not yet been reported. Natural inducers of xylanolytic genes in *Aspergillus* may be products of xylan degradation or transglycosylation processes, such as D-xylose, xylobiose, xylotriose, and xylotetrose [27]. However, in the presence of readily metabolizable carbon sources such as D-glucose, gene expression involved in the use of less-favored carbon sources, such as the xylanolytic system, is inhibited due to carbon catabolite repression [21]. We describe the design of a system capable of producing β-xylosidase even in presence of its repressor. The purpose of our study was to constitutively express and partially characterize a β-xylosidase from the native strain *A. niger* GS1 by means of a fungal system.

Materials and methods

Materials

All chemicals were of analytical grade and were purchased from Sigma (St. Louis, MO, USA), except as indicated.

Microorganisms and plasmid

Aspergillus niger GS1 (NCBI No. GU395669) was used as a source of xylosidase gene (UAQ, Queretaro, Mexico). Spores isolated from *A. niger* GS1 were stored in Tween 20 on silica gel at 4°C. Stock cultures were subcultured on fresh sterile potato dextrose agar (PDA; Bioxon, Cuautitlán, Mexico) plates and incubated for 72–120 h at 30°C [23]. *Escherichia coli* JM109 genotype recA1, endA1, gyrA96, thi, hsdR17 (rk-mK+), relA1, supE44, Δ(lac-proAB) [F', traD36, proAB, lacIqZΔM15] (Promega, Madison, WI, USA) was used to propagate vectors and was cultured at 37°C in Luria–Bertani medium comprising (g l^{-1}): bacto-tryptone (Difco, Franklin Lakes, NJ, USA), 10; yeast extract (Difco), 5; sodium chloride (NaCl), 10; supplemented with 100 μg ml^{-1} ampicillin. The pGEM-T plasmid (Promega) was used as the subcloning vector, and *A. niger* AB4.1 (*pyrG*$^-$) strain [29] was used for homologous expression of xylosidase (*xlnD*) gene. Vector pAN52.1 was used to construct the constitutive expression vector pANJil. This vector contains the *gpdA* promoter and the terminator region of the *trpC* gene (both from *A. nidulans*) separated by *Bam*HI and *Nco*I sites. Vector pAB4.1 (*pyrG*) [29] was used as selection marker. Both vectors were kindly provided by Dr. Punt (TNO, The Netherlands).

Induction of β-xylosidase

Aspergillus niger GS1 spores were inoculated into PDA-xylan slants (glucose 15 g l^{-1}, oat spelts xylan 5 g l^{-1}, agar–agar (Bioxon) 15 g l^{-1}, and potato infusion 0.5 l), pH 5.5–6.0, incubated at 30°C, for 72 h. Harvested spores were then transferred to PDA-xylan slants increasing by 5 g l^{-1} the initial xylan concentration while decreasing initial glucose concentration by 5 g l^{-1} until complete replacement with xylan as main carbon source (g l^{-1}) (agar–agar, 15; oat spelts xylan, 20; and potato infusion, 0.5 l) was attained. Spores collected from the potato xylan agar were seeded in basal xylan media supplemented with yeast extract (oat spelts xylan 25 g l^{-1}, yeast extract 5 g l^{-1}). After incubation for 2–3 days, mycelia were collected for RNA extraction using the RNeasy Plant Mini Kit (Qiagen, Hamburg, Germany).

Molecular identification of *A. niger* GS1, and *xlnD* gene

Aspergillus niger GS1 was genetically identified by sequencing the 26S ribosomal DNA (rDNA) (GBC-IPN, México). Collected mycelia from basal xylan media was employed for RNA extraction. Forward (JilF 5′-CCATG GATGGCGCACTCAATGTCTCG-3′), and reverse (JilR 5′-CTGGATCCCTA*GTGGTGATGGTGATGATG*CTCCT TCCCCGGCCAC-3′) primers were designed using the National Center for Biotechnical Information (NCBI)-reported sequence (ANXM_001389379) for *A. niger* β-1,4-xylan xylosidase. Bases coding for His-tag are shown in italics.

The RevertAidH Minus kit (Fermentas, Ontario, Canada) was used to obtain xylosidase complementary DNA (cDNA) from total RNA (tRNA) (2 μg) following the manufacturer's instructions. Polymerase chain reaction (PCR) amplification of *A. niger* GS1 cDNA (100 ng) was conducted using 400 mM primers; 10 mM deoxyribonucleotide triphosphate (dNTP), 1.5 mM magnesium chloride ($MgCl_2$), 1× reaction buffer [200 mM Tris pH 8.4, 500 mM calcium chloride (KCl)] (Invitrogen, Carlsbad, CA, USA), and 2 U recombinant *Taq* polymerase (Invitrogen) in a final volume of 50 μl. Reaction conditions were 5 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 65°C, 2.5 min at 72°C, and a final extension of 10 min at 72°C. The amplicon was ligated into pGEM-T vector, followed by heat-shock cloning into *E. coli* JM109. Clones containing the insert were directly identified by blue/white color screening on indicator plates, and then from Miniprep (Qiagen) extraction, the isolated plasmid was sent for sequencing (MCLab, San Francisco, CA, USA). After sequence confirmation, the DNA open-reading frame was cloned into the expression vector pAN52.1 (cloning sites *Nco*I and *BamH*I) to obtain pANJil expression vector.

Aspergillus transformation

Fungal cotransformation was accomplished using the procedure reported by Sanchez and Aguirre [25] developed for *A. nidulans,* with modifications. Spores from *A. niger* AB4.1 were washed with 10 ml sterile distilled water, and an inoculum of 8.6×10^6 spores ml^{-1} was added to 50 ml of dextrose potato broth (Difco) supplemented with uridine (2.5 g l^{-1}), followed by incubation at 30°C in a rotary shaker (300 rpm) for 15 h. Next, germinating spores (GTS) were recovered by centrifugation at 4,000×*g* for 10 min at 4°C (Eppendorf, Mod. 5804R, Hamburg, Germany). GTS were resuspended in 50 ml of ice-cold sterile water, centrifuged again, resuspended in 25 ml of ice-cold pretreatment buffer [1% yeast extract, 1% glucose; YED] plus 20 mM hydroxyethyl-1-piperazine ethanesulfonic acid (HEPES) (adjusted to pH 8.0 with 100 mM Tris), and incubated for 1 h at 30°C in a rotary shaker at 100 rpm. After this incubation, GTS were centrifuged and resuspended in 1 ml (about 2.2×10^7 spores ml^{-1}, final) of ice-cold electroporation buffer [10 mM Tris–HCl(pH 7.3), 270 mM sucrose, 1 mM HEPES, 10 mM rubidium chloride (RbCl), 10 mM lithium chloride (LiCl)], kept on ice, and stored at −70°C. For electroporation, 2 μg of total DNA (tDNA) (pANJil expression vector with pAB4.1 vector in a 10:1 volume ratio) was added to 50 μl of ice-cold GTS suspension. The mixture was then incubated on ice for 15 min and transferred to a 0.2-cm cuvette. Electroporation was performed using a MicroPulser Electroporation Apparatus (Bio-Rad, Hercules, CA, USA), adjusting voltage to 1.4 kV and pulses lasting approximately 3.5 ms. After electroporation, 600 μl of ice-cold YED was added to the cuvette and the cell suspension transferred to a sterile 1.5-ml tube, kept on ice for 10 min, and incubated at 30°C for 90 min in a rotary shaker at 100 rpm. Electroporated spores (100 μl $plate^{-1}$) were extended on sorbitol-containing minimal agar (g l^{-1}): glucose, 10; sorbitol, 218.64; sodium nitrate ($NaNO_3$), 6; KCl, 0.52, potassium dihydrogen phosphate (KH_2PO_4), 1.52; trace elements: zinc sulfite ($ZnSO_4$ $7H_2O$), 0.022; boric acid (H_3BO_3), 0.011; manganese (II) chloride ($MnCl_2$ $4H_2O$), 0.005; ferrous sulfate ($FeSO_4$ $7H_2O$), 0.005; cobalt(II) chloride ($CoCl_2$ $6H_2O$), 0.0017; copper(II) sulfate ($CuSO_4$ $5H_2O$), 0.0016; sodium molybdate (Na_2MoO_4 H_2O), 0.0015; sodium ethylenediaminetetraacetate (Na_2EDTA) 0.05; without uridine. Spores were incubated at 30°C, for 48 h. The stability of uridine prototroph transformants was tested by velvet-replica plating on selective minimal medium. In addition, a control was transformed with the *pyrG* gene but without expression vector.

Screening and production of β-xylosidase activity

Cotransformants were plated on the selective minimal medium (without uridine) and incubated for 8 days at 30°C. Liquid cultures were inoculated in glucose-rich medium (g l^{-1}): glucose, 20; yeast extract, 0.5; $NaNO_3$, 7.5; $(NH_4)_2SO_4$, 1.5; KCl, 8.67; $MgSO_4$ $7H_2O$, 8.67; and trace elements) with 2×10^6 spores ml^{-1} in 50-ml Falcon tubes. To screen cotransformants, up to 20 individual clones were inoculated into minimal medium and checked daily for xylosidase activity for 4 days. For the activity assay, a 1-ml aliquot of culture medium was collected, and cells were removed by filtration through a 0.45-μm filter (Millipore, Billerica, MA, USA). Positive cotransformants were assayed through JilF–JilR amplification using genomic DNA as template, obtained by the cetyl trimethylammonium bromide (CTAB) protocol [1].

Analytical methods

Protein and β-xylosidase activity

Soluble protein content was determined according to Bradford [2] using bovine serum albumin (BSA) as standard. Xylosidase activity was measured by the *p*-nitrophenol method, as described by Pedersen et al. [18], using 2.5 mM *p*-nitrophenol-β-D-xylopyranoside (PNPX) in 50 mM acetate buffer and 100 μl of enzyme solution at pH 4.0 in a total reaction volume of 1 ml. After incubation at 50°C for 10 min, the reaction was stopped by adding 1 M Na_2CO_3 (JT Baker, Phillipsburg, NJ, USA) to a final concentration of 0.33 mM, and the released *p*-nitrophenol

was measured spectrophotometrically at 400 nm. One unit of β-xylosidase activity was calculated as the amount of enzyme producing 1 µmol equivalent of *p*-nitrophenol per min, as calculated from a standard curve. Endo-β-1,4-xylanase and cellulase activities of the purified extract was determined using 5 g l^{-1} xylan and carboxymethyl cellulose as substrates, respectively, dissolved in 50 mM acetate buffer, pH 5.5. The reaction mixture consisted of 100 µl of enzyme solution, 400 µl of the corresponding substrate, and incubated at 50°C for 10 min, followed by immersion in ice-cold water. Released reducing sugars were quantified according to Miller [15] using a xylose and a glucose standard curve (1–10 mM). One activity unit (U) was defined as the amount of enzyme that released 1 µmol of xylose or glucose equivalents per minute at 50°C.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) was carried out using a 10% (w/v) T (% acrylamide plus bis-acrylamide in gelling solution) according to Laemmli [11], and protein bands were stained with Coomassie Brilliant Blue R-250 [1]. β-xylosidase activity was detected in the gel after electrophoresis by cutting bands. Each band was washed three times with high-performance liquid chromatography (HPLC)-grade water, placed in a microtube containing 200 µl substrate solution (PNPX), and incubated for 30 min at 50°C. The reaction was stopped by adding 150 µl of 1 M Na_2CO_3 solution, and absorbance was measured at 400 nm.

Purification of the recombinant β-xylosidase

To purify the recombinant β-xylosidase, cotransformed *A. niger* was cultivated in glucose-rich medium. Then, 600 ml of a 4-day-old culture medium was filtered through Whatman No. 4 (Whatman International, Maidstone, UK) and 0.2 µm membrane (Millipore) and concentrated using a Centricon centrifugal filter unit with molecular mass cutoff membrane of 10 kDa (Millipore). Concentrated enzyme (2.0 ml) was applied into a diethylaminoethanol (DEAE)-cellulose column (1.5 × 25 cm), equilibrated, and washed with 50 mM acetate buffer, pH 3.9, at a flow rate of 25 ml h^{-1}. Bound proteins were eluted using the same washing buffer plus 1 M NaCl. Fractions of 1.5 ml were collected and assayed for absorbance at 280 nm and β-xylosidase activity, and those showing high activity were pooled and concentrated using the Centricon units (10 kDa). Concentrated fractions were then loaded onto a Nickel (Ni) Sepharose 6 Fast Flow column (1 ml) (GE Healthcare Bio-sciences, Uppsala, Sweden) following the manufacturer's manual.

Effect of temperature and pH on purified recombinant β-xylosidase activity

To determine optimal temperature of purified recombinant β-xylosidase, activity determinations were conducted using 65-ng aliquots of purified enzyme and incubating at 30–80°C. Activation energy (Ea) of recombinant β-xylosidase was calculated following an Arrhenius-type behavior by plotting ln (activity) vs (absolute temperature, K)$^{-1}$, in the range of 30° to 60°C. The slope of this plot indicates (–Ea R^{-1}), where *R* is the universal gas constant. Optimal pH was determined using same protein aliquots of the enzyme in same total reaction volume, and activity was measured using acetate buffer (50 mM) for pH values 3.6–5.0; 50 mM phosphate buffer was used for pH values 6.0–8.0. Experiments were conducted for three replicates.

Temperature and pH stability of the purified recombinant β-xylosidase

Aliquots (65 ng) of purified recombinant β-xylosidase were preincubated in the range of 50° to 70°C for 5, 60, and 120 min using an AccuBlock dry bath (Labnet, Edison, NJ, USA). After cooling at 4°C, xylosidase activity was assayed. The recombinant enzyme half-life was calculated by linear regression analysis of specific activity at desired temperature against time. The effect of pH on the purified recombinant enzyme stability was studied by incubating in 50 mM acetate buffer (pH 4.0, 5.0) and 50 mM phosphate buffer (pH 6.0), at 10 ± 2°C for 2 and 24 h, followed by activity determination. Experiments were conducted for three replicates.

Effect of metal ions and chemical reagents on recombinant β-xylosidase activity

The effects of several metal ion salts and chemicals on the recombinant purified β-xylosidase were tested by measuring enzyme activity in the presence of individual compounds at a final concentration of 10 mM. These chemicals have been shown to affect xylanolytic activity [6, 8], and those used were $CuSO_4$ $5H_2O$, LiCl, $ZnCl_2$, Na_2EDTA, SDS, dithiothreitol (DTT), and β-mercaptoethanol. After preincubating for 10 min at room temperature (26 ± 2°C), the residual enzyme activities were expressed as the percentage of enzyme activity without added chemical.

Results and discussion

Molecular identification of *A. niger* GS1 and *xlnD* gene

Aspergillus niger GS1 was isolated from copra paste. A pure culture was obtained by single streaking on PDA and

morphologically identified by microscopic visualization of its reproductive structures and main characteristics [23]. This native Mexican fungal strain was genetically identified by sequencing the 26S rRNA gene. Sequence of this gene has been extensively used to establish phylogenetic and systematic relationships within fungi [13]. A partial sequence of 552 bp was obtained and submitted to the NCBI database, with accession No. GU395669. Blast analysis against NCBI genome database showed 100% similarity with *A. niger* CBS 513.88 clone An03 (NT_166520.1). In addition, using the nucleotide collection, a 99% identity was found for partial sequence of 26S RNA genes of *A. niger* VTCC:F021 (GQ382274.1), and *A. niger* VTCC:CF128 26 S (GQ382273.1). Thus, from both morphological and molecular analysis, we conclude that the isolated strain was *A. niger*. A single amplification band was obtained from PCR products with an approximate size of 2,433 bp when primers JilR and JilF were used (data not shown). PCR product was inserted into pGEM-T vector, and positive clones identified by sequencing showed up to 99% homology with *A. niger* CBS 513.88 xylosidase (*xlnD*) (XM_0013819379) partial messenger RNA (mRNA). Our sequence corresponded to a complete structural gene coding for β-xylosidase, plus a six histidine tag before the stop codon, which was submitted to NCBI database, obtaining accession number GU585573. In addition, our findings are in agreement with La Grange et al. [10], who reported that β-xylosidase in *A. niger* is coded by *xlnD*, containing an open reading frame of 2,412 nucleotides, which encodes a theoretical protein of 804 amino acids (85 kDa).

Expression of β-xylosidase gene from the *gpdA* promoter

To avoid catabolic repression exerted by glucose over *xlnD* expression, we expressed the β-xylosidase from *A. niger* GS1 into pAN52-1 under the control of the strong constitutive *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) promoter and *trpC* terminator. The *gpdA* promoter is expressed efficiently in *A. niger* strains [7, 17]. The construction of pANJil included a DNA sequence encoding the first 26 amino acids of the *xlnD* signal peptide (ALA-QA), according to Bendtsen et al. [3]. The pANJil was introduced into *A. niger* AB4.1 strain by cotransformation with the plasmid pAB4.1, which contains the *A. nidulans pyrG* gene encoding orotidine 5′-phosphate carboxylase. Preliminary analysis of Pyr^+ regenerants from these cotransformations identified up to 200 transformants. From those transformants, 15 were streaked twice on minimal plates and then screened for β-xylosidase activity on minimal liquid media under glucose-rich conditions (20 g l^{-1}) that repress *xlnD* synthesis in the wild-type strain. Two strains (gpd-Jil-1, -2) that were morphologically stable showed β-xylosidase activity and were cultured for genomic DNA isolation, which was analyzed by PCR using JilF and JilR primers. One strain, designated gpd-Jil-1, showed a single-band amplification pattern of about 2,433 bp (Fig. 1), corresponding to the reported *xlnD* open-reading frame in *A. niger* (AN Z84377); on the other hand, no band pattern was found in the control strain (pAB4.1 single transformant). From 13 selected possibly cotransformed strains, only two showed this sequence upon PCR amplification using the above-mentioned primers. Thus, it is possible that the recipient strain had a sequence variant for this gene, as previously shown [19]. However, added His-tag plus *Bam*H1 site sequence (29 bp) is not naturally occurring and therefore would not be amplified under the stringent conditions used in this study unless there were fully cotransformed cells.

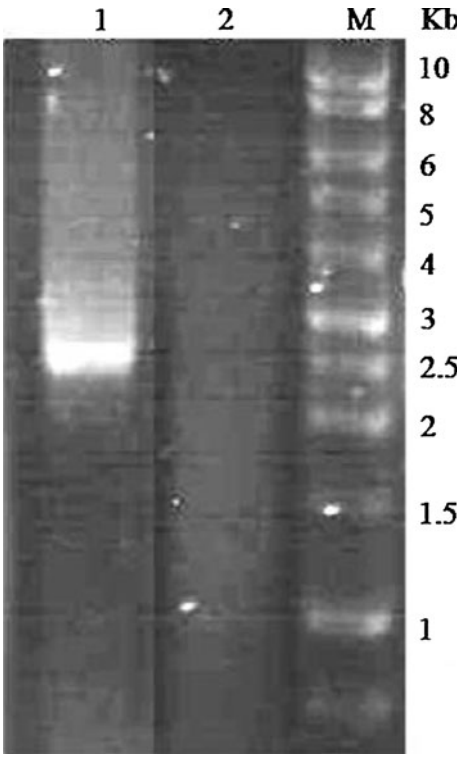

Fig. 1 Agarose gel (1% w/v) visualization of polymerase chain reaction (PCR) products amplified with JilF and JilR primers using cotransformed genomic DNA of *Aspergillus niger* gpd-Jil-1 as template. *Lanes*: *M*, 1 kb DNA ladder (Promega); *1* amplicon from gpd-Jil-1 using primers Jil-F and Jil-R; *2* PCR from single transformant using primers Jil-F and Jil-R

Purification of the recombinant β-xylosidase

Preliminary experiments showed that *A. niger* GS1 produced a complex of hemicellulolytic enzymes (mannanase, cellulose, xylosidase) after solid-state fermentation. These

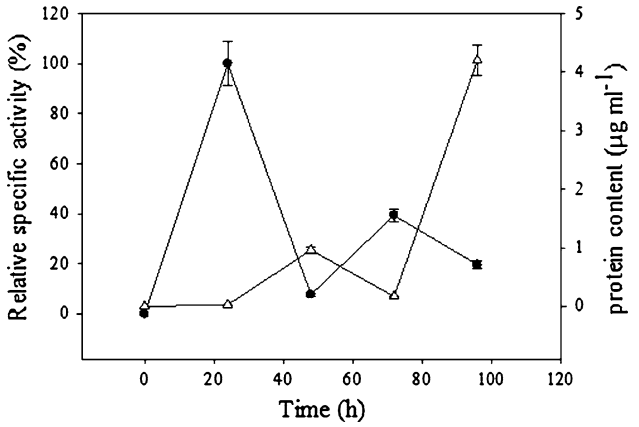

Fig. 2 Relative (i.e., ratio of actual to maximum) β-xylosidase specific activity (*filled circles*) and protein concentration (*open triangles*) in fermentation broth by growing *Aspergillus niger* gpd-Jil-1 in glucose-rich medium. Each *data point* represents the mean of three independent experiments ± standard deviation

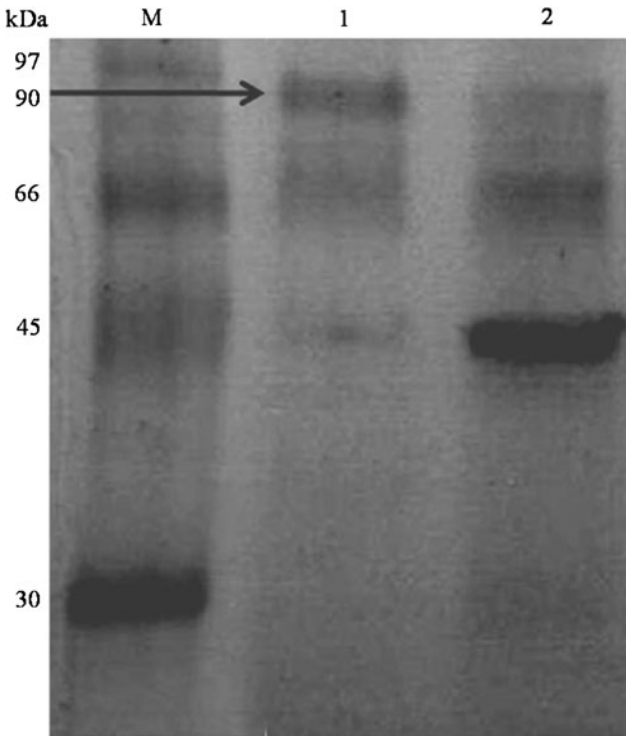

Fig. 3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of purified β-xylosidase from *Aspergillus niger* gpd-Jil-1. *Lanes*: *M*, molecular weight markers: rabbit muscle phosphorylase b (97 kDa), bovine serum albumin (66 kDa), chicken egg white ovalbumin (45 kDa), bovine erythrocyte carbonic anhydrase (30 kDa) (GE Healthcare); *1* purified recombinant β-xylosidase; *2* culture supernatant proteins

results indicate that hemicellulolytic enzyme expression is subject to induction and carbon catabolic repression, according to substrate composition used for fermentation (data not shown). Therefore, the choice of an appropriate system to constitutively provide hemicellulolytic free β-xylosidase activity without being affected by catabolic repression is of importance for processes such as modified xylooligosaccharides production. Growth of our cotransformed strain in glucose-rich medium produced steadily increased β-xylosidase activity up to 24 h but showed a strong decrease after 48 h and remained constant up to the end of fermentation time (96 h) (Fig. 2). This behavior may be due to the presence of proteases (data not shown), despite the fact that protein content kept increasing during fermentation (Fig. 2). Furthermore, small β-xylosidase activity that might be associated with the cell wall was found from acetate buffer (50 mM, pH 3.6) extracts of crushed mycelia (832 U mg protein^{-1}). Enzyme activity was not detected in any of the PyrG^{+} transformants. This is in agreement with the behavior reported for other recombinant β-xylosidases [19].

The transformant *A. niger* gpd-Jil-1 produced one recombinant β-xylosidase, and from culture supernatant it was purified 31.6-fold through the Ni-affinity chromatography column, with 9% activity recovery. Purified β-xylosidase showed a specific activity of 2,094 U mg protein^{-1} at 50°C and pH 4, and showed no endo-β-xylanase or cellulase activities when tested with xylan or carboxy-methylcellulose (CMC) as substrates, respectively. SDS–PAGE showed a protein band with an apparent molecular weight of 90 kDa (Fig. 3), which was the only one showing β-xylosidase activity after testing each band.

The purified β-xylosidase showed a size similar to those reported for other *Aspergillus* spp. (62–122 kDa) [9, 30]. A wide range of purified β-xylosidase activity have been reported for *Aspergillus* spp., with the highest, isolated from tannins, showed 55,800 U mg protein^{-1} [18], which is 27 times more active than that obtained in this study (assay as depicted in section "Protein and β-xylosidase activity"). Other authors have reported lower activities, such as 360 U mg protein^{-1} [12], for a xylosidase isolated from a thermotolerant *A. fumigatus.* On the other hand, a xylosidase from *A. japonicus* showed 112 U mg protein^{-1} [30], whereas its recombinant counterpart showed only 18.7 U mg protein^{-1}, which is 112 times lower than the activity found in our study.

Effect of temperature on activity and stability of recombinant β-xylosidase

The temperature for maximum activity of the recombinant β-xylosidase was 70°C, whereas at 50° and 60°C, the enzyme exhibited 57.5% and 93.6% of maximum activity, respectively (Fig. 4.). These results are in agreement with those previously reported, indicating the thermally stable nature of *A. niger* XlnD [18, 26]. It was evident that the

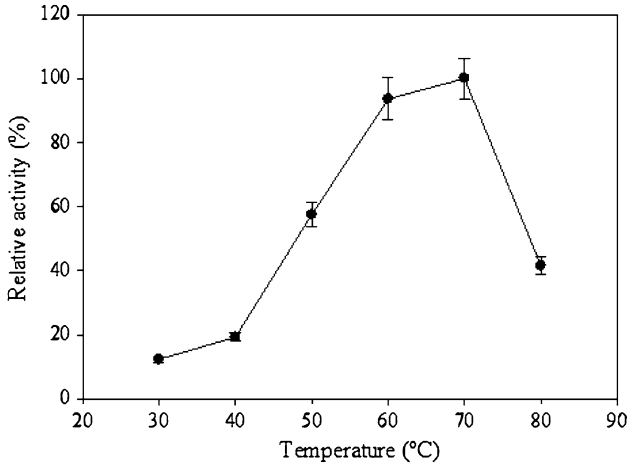

Fig. 4 Effect of temperature on activity of recombinant β-xylosidase. The *ordinate* represents relative activity that is the ratio of the activity to the activity found at optimal temperature (70°C, 3,272 U mg protein^{-1}) expressed as percentage. Each *data point* represents the mean of three independent experiments ± standard deviation

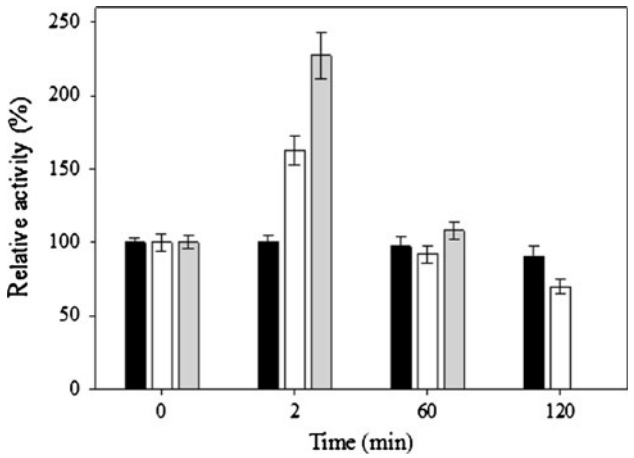

Fig. 5 Thermostability of recombinant β-xylosidase at 50°C (*filled square*), 60°C (*open square*), and 70°C (*gray shaded square*). The *ordinate* represents relative activity that is the ratio of the activity to the initial activity (1,883 U mg protein^{-1}) expressed as percentage. Each *data point* represents the mean of three independent experiments ± standard deviation

expression system used in this work was successful in retaining protein thermal stability.

Thermostability studies showed that the purified enzyme was stable at 50°C for 2 h, whereas a relatively long enzyme half-life (74 min) was observed at 70°C (Fig. 5). After incubation at 80°C for 10 min, purified xylosidase showed activity slightly lower than that of the enzyme heated at 50°C for same time (Fig. 4). This property may be useful in applications where high temperatures are required for short time, e.g., extrusion processes.

Optimal temperature of a purified β-xylosidase from *A. phoenicis* was 75°C [24], in agreement with our results. However, thermal stability was smaller than that shown by our recombinant enzyme because residual activity after 1 h at 65°, 70°, and 75°C significantly decreased (<30%, 25%, and 0%, respectively). On the other hand, recombinant β-xylosidase from *A. niger* showed 80% remaining activity after incubating at 55°C for 2 h. However, thermal stability was lower than that of our recombinant enzyme because 60% activity reduction was found by incubating at 60°C for 20 min, and after 2 h, activity decreased to <10% [10]. Only a few *A. niger* β-xylosidases are reported to be active and stable at high temperatures [18, 26], whereas similar findings are reported for *A. phoenicis*, which retained 100% activity after 4 h at 60°C [20].

Following Arrhenius type behavior, a determination coefficient (r^2) of 0.97 was obtained, and from the slope, the activation energy was 58.9 kJ mol^{-1}. This value is similar to that reported for a β-xylosidase from *Thermoanaerobacter ethanolicus,* which was 69 kJ mol^{-1}. Prediction electronic tools for O-linked β-N-acetylglucosamine (O-GlNAc) and O-β-GlcNAc attachment sites in eukaryotic protein sequences, OGPET v. 1.0 (http//www.ogpet.utep.edu/ogpet/index.php), and YinOYang v. 1.2 (http//www.cbs.dtu.dk/services/YinOYang) respectively, using β-xylosidase amino acid sequence analysis showed 14 potential glycosylation sites. The carbohydrate moiety of β-xylosidase from most A*spergillus* spp. has been estimated to be between 10% and 47%, which promotes further enzyme stability under denaturing conditions without affecting catalytic activity [9].

Effect of pH on activity and stability of recombinant β-xylosidase

The pH of maximum activity of purified recombinant β-xylosidase was around 3.6 (Fig. 6). Activity decreased to 85.6, 74.9, and 44.4% of the optimum at pH values 4.0, 5.0, and 6.0, respectively. This is in contrast with the results found for β-xylosidase from *A. niger* IBT-3250, where activity was similar in the range 3.0–5.0 [18]. The optimum pH value was similar to that found for a β-xylosidase from *A. pulverulentus* (2.5–3.5), which shows a versatile feature in these strains, as the majority of other *Aspergillus* spp. produce β-xylosidases with optimal pH between 4.0 and 6.0 [5]. Using the optimum pH (3.6) and temperature (70°C), the recombinant β-xylosidase activity was found to be as high as 4,280 U mg protein^{-1}.

Results of stability of purified β-xylosidase toward pH indicate that it was fairly stable after 2 h incubation at pH values of 4.0 and 5.0 (Fig. 7). Nevertheless, the activity slightly decreased at pH 6.0 (58.4% of its initial activity). After 24 h of recombinant enzyme incubation at 10 ± 2°C, the lowest relative activity was 52.1% at pH 6.0 (Fig. 7). On the other hand, a β-xylosidase from *A. phoenicis* was stable over the pH range 4.0–6.0 for 7 h at room temperature [24].

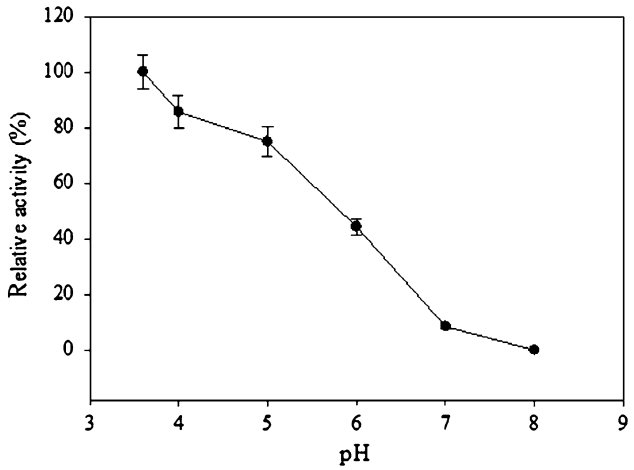

Fig. 6 Effect of pH on recombinant β-xylosidase activity. Acetate buffer (50 mM) was used for pH 3.6, 4.0, and 5.0; 50 mM phosphate buffer was used for pH 6.0, 7.0, and 8.0. The *ordinate* represents relative activity that is the ratio of the activity to the activity found at optimal pH (2,694 U mg protein^{-1}) expressed as percentage. Each *data point* represents the mean of three independent experiments ± standard deviation

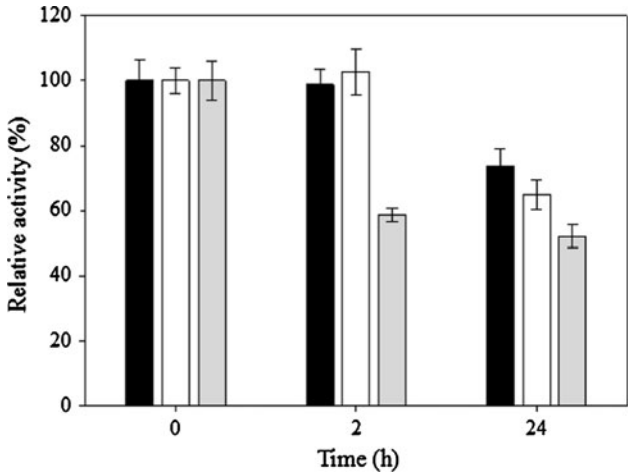

Fig. 7 Effect of pH on recombinant β-xylosidase stability. The enzyme was incubated at 10°C for 2 h and 24 h. Acetate buffer (50 mM) was used for pH 4.0 (*filled square*) and pH 5.0 (*open square*), whereas 50 mM phosphate buffer was used for pH 6.0 (*gray shaded*). The *ordinate* represents relative activity that is the ratio of the activity to initial activity (2,137 U mg protein^{-1}). Each *data point* represents the mean of three independent experiments ± standard deviation

In addition, a recombinant β-xylosidase from *A. japonicus* retained >90% of its original activity between pH 2.0 and 7.0 when incubating at room temperature for 3 h [30].

Effect of metal ions and chemicals on recombinant β-xylosidase activity

Effects of various metallic ions and other reagents on the activity of purified β-xylosidase were investigated. As shown in Table 1, activity was dramatically inhibited (58.5% and 72.5% of residual activity) by Cu^{2+} and Li^{+}. A slight inhibition (around 83%) was observed in the presence of EDTA, SDS, and β-mercaptoethanol, whereas residual activity was 91.3% in the presence of DTT. β-xylosidases from *Emericella nidulans* and *A. nidulans* showed some resistance to SDS but high sensitivity to Cu^{2+} [8]. The author of that study also reported a stimulating effect on residual activity when EDTA, DTT, and Zn^{2+} where added, which is in contrast to the behavior reported here. An effect similar to the one found here was noted by Rizzati [24], who used a purified β-xylosidase from *A. phoenicis,* when Cu^{2+}, EDTA, and β-mercaptoethanol were added, but an opposite effect was observed on activity when Zn^{2+} was added.

Table 1 Residual activity of recombinant β-xylosidase after incubating for 10 min at 28°C with different additives (10 mM each)

Added chemical	Residual activity (%)
No additive	100[a]
Cu	58.5 ± 11[b]
Li	71.3 ± 3.3
Zn	107 ± 14
EDTA	85.3 ± 9.7
SDS	85.3 ± 11
DTT	91.3 ± 11
β-mercaptoethanol	83.8 ± 8.5

Cu copper, *Li* lithium, *Zn* zinc, *EDTA* ethylenediaminetetraacetate, *SDS* sodium dodecyl sulfate, *DTT* dithiothreitol

[a] 100% residual activity corresponded to 2,194 U mg protein^{-1}. Values represent mean of three independent experiments

[b] Unstable solution, showing small precipitate

Retention of main activity by β-xylosidase (>83%) in the presence of reducing agents, detergents, and some salts, such as DTT, β-mercaptoethanol, SDS, EDTA, and Zn^{+} show another remarkable feature of this enzyme, which represents an advantage in industrial applications where the presence of these compounds is unavoidable.

Conclusion

This work successfully obtained hemicellulolytic-free xylosidase when growing *A. niger* gpd-Jil-1 using a single carbon source. Recombinant β-xylosidase produced by *A. niger* gpd-Jil-1 exhibited significant activity at high temperatures, in acid media, and in the presence of reducing agents. It is thus likely to have good potential in animal feed, enzymatic synthesis, and the fruit-juice industry.

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