Isolation and properties of β-xylosidase from *Aspergillus niger* GS1 using corn pericarp upon solid state fermentation

F.I. Díaz-Malváez[a], B.E. García-Almendárez[a], A. Hernández-Arana[b],
A. Amaro-Reyes[a], C. Regalado-González[a,*]

[a] DIPA, PROPAC. Facultad de Química, Universidad Autónoma de Querétaro, C.U. Cerro de las Campanas s/n. Col. Las Campanas, C.P. 76010 Querétaro, Qro., Mexico
[b] Departamento de Química, Universidad Autónoma Metropolitana-Iztapalapa, Av. San Rafael Atlixco No. 186, Col. Vicentina, Delegación Iztapalapa, C.P. 09340 México, D.F., Mexico

ARTICLE INFO

ABSTRACT

There is growing interest in developing high-yield and low-cost production of xylanolytic enzymes for industrial applications using agroindustrial byproducts. A native strain of *Aspergillus niger* GS1 was used to produce β-xylosidase (EC 3.2.1.37) on solid state fermentation using corn pericarp (CP) with innovative alkaline electrolyzed water (AEW) pretreatment at room temperature. β-xylosidase was purified by ammonium sulfate fractionation followed by anion exchange and hydrophobic interaction chromatographies. β-Xylosidase showed a molecular weight of 111 kDa, isoelectric point of 5.35 and specific activity of 386.7 U (mg protein)$^{-1}$, using *p*-nitrophenyl-β-D-xylopyranoside as substrate, at pH 5 and 60 °C, and optimal activity at pH 4.5. Optimal temperature was 65 °C, showing full activity after 1 h at 60 °C. Activity was reduced by 1 mM β-mercaptoethanol (55.6 ± 0.1%), and enhanced by 1 mM SDS (11.0 ± 0.03%). K_m and V_{max} were 6.1 ± 0.9 mM and 1364 ± 105 U (mg protein)$^{-1}$, respectively, whereas k_{cat} was 5.1 s^{-1}. A predominant α-helix (41%) was determined from circular dichroism on β-xylosidase, while thermal transition profiles produced a T_m of 54.1 ± 5.8 °C, enthalpy change for unfolding of 67.4 ± 6.7 kJ/mol, and onset temperature of 37 °C. Pre-treatment of CP using AEW is an ecologically friendly alternative to chemical and heat treatments for the production of relatively high levels of β-xylosidase.

© 2013 Elsevier Ltd. All rights reserved.

1. Introduction

Hemicellulose is the second most common polysaccharide in nature and represents about 20–35% of lignocellulosic biomass. Xylans are the major portion of plant cell walls hemicellulose, and are heteropolymers containing mainly xylose and arabinose [1,2]. Agroindustrial residues such as corn pericarp (CP), cobs and stubble; wheat, and rice straw contain about 20–40% hemicellulose [3]. CP is a byproduct from the wet milling industry and represents 5.3% of whole corn kernel, comprising up to 70% hemicellulose [4]. CP is highly available in Mexico (~0.1 million tons year^{-1}) and may provide a low cost feedstock. However, high value added biological products such as enzymes, fuels and chemicals, may be produced with environmental and strategic advantages [5]. Due to heterogeneity and complex chemical nature of plant xylans, several hemicellulolytic enzymes with diverse specificities and modes of action are needed for their complete breakdown. β-xylosidase (EC 3.2.1.37) in synergistic action with endo-β-xylanases and debranching enzymes namely α-glucuronidases, esterases and glycosidases have potential applications; food (fruit, vegetable processing, brewing, wine production, baking), feed (animal feed), technical (paper and pulp, textile, bioremediation/bioconversion) and biofuel (second generation biofuels) industries. Important applications have been attributed to thermophilic hemicellulases, such as processes where high temperatures are required to increase bioavailability and/or solubility of substrates, to reduce viscosity and/or to reduce risk of contamination [3,6–8]. β-xylosidase hydrolyses xylobiose and xylooligosaccharides to xylose from the non-reducing end [9,10]. Filamentous fungi secrete β-xylosidases into the medium and are thus particularly interesting producers of this enzyme from an industrial point of view [10,11]. Several fungi such as *Trichoderma reesei, Aspergillus niger* and *Penicillium* sp. have been employed for hemicellulases production using solid-state fermentation (SSF) in which basal mineral salts medium is used for moistening the substrate [12,13]. SSF is defined as that occurring in absence or near absence of free water [14]. However, the substrate must possess enough moisture to support growth and metabolism of microorganisms and it has recently gained importance for the production of microbial enzymes due to several economic advantages over conventional submerged fermentation [13]. Recently, acidic and alkaline electrolyzed water have been used as pretreatment for partial removal of plant cell wall complex structures followed by commercial enzymatic treatment

* Corresponding author. Tel.: +52 442 1921307.
  E-mail addresses: carlosr@uaq.mx, regcarlos@gmail.com
(C. Regalado-González).

for biofuels production [15–19]. In all studies substrate pretreatment was successful at temperatures >170 °C suggesting expensive pressure tight reactors and a high energy process involved. The molecular weight of β-xylosidases ranges 37.5–360 kDa and can be monomeric, dimeric, or trimeric which most from fungi show molecular weights above 100 kDa, and those from *A. niger* exhibit optimum pH in the range 5–6.5 [10,20]. Based on their primary sequence, β-xylosidases have been grouped into different families (CAZy database, http://www.cazy.org) the so-called glycoside hydrolases GH 3, 30, 39, 43, 52 and 54 [13,15,21]. The majority of the isolated and characterized β-xylosidases are optimally active at temperatures ranging 25–40 °C, hampering their application as robust tools for the industry, bearing in mind that xylan degradation products have the potential as energy source in the future [10].

The aim of this work was to isolate and evaluate the properties of a new β-xylosidase produced from *Aspergillus niger* GS1, using solid state fermentation on alkaline electrolyzed water (AEW) pretreated CP.

2. Materials and methods

2.1. Materials

All chemicals were of analytical grade and were purchased from Sigma (St. Louis, MO, USA), except as indicated. CP was obtained from Ingredion, San Juan del Río, Querétaro, México. AEW containing NaOH = 0.55–0.60 g L^{-1}, pH = 11.5–12.5 (Limy®) was provided by Grupo EcoRus AEQ, Mexico.

2.2. Microorganism

Aspergillus niger GS1 isolated from copra paste and molecularly identified (NCBI No. GU 95669) [22] was used as a source of β-xylosidase. It was propagated at 30 °C for seven days in potato dextrose agar. Inoculum was prepared by suspending the conidia from PDA by adding sterile 0.1% Tween-80 solution.

2.3. Substrate and solid state fermentation

CP was washed with distilled water and treated with AEW diluted in a 1:4 ratio with distilled water, achieving 150 ppm NaOH, pH 9.2, contacted for 5 h at room temperature (25 ± 2 °C) with gentle stirring followed by rinsing with distilled water, dried and milled (Cemotec, Tecator, Hillerød, Denmark). The resulting powder was separated by sieving and particle size in the range 0.40–1.40 mm was used. CP was analyzed for moisture (gravimetrically) [23], crude protein (Nx6.25) [24], ether extract [25], and ash [26]. Hemicellulose, cellulose and lignin (organic matter based) were determined from neutral (NDF) [27] and acid (ADF) detergent fibers [28] using an ANKOM 200 digestion equipment (ANKOM Technology, Macedon, NY, USA). Preliminary experiments varying carbon and nitrogen sources concentration as well as moisture content did not produce better results than those obtained from previously optimized experimental conditions [29]. Thus, the nutritional supplement used contained yeast extract 16.6 g L^{-1}, (NH$_4$)$_2$SO$_4$ 1.44 g L^{-1}, glucose 8.43 g L^{-1}, Na$_2$HPO$_4$ 4 g L^{-1}, MgSO$_4$·7H$_2$O 0.5 g L^{-1}, FeSO$_4$·7H$_2$O 0.31 g L^{-1}, KH$_2$PO$_4$ 2 g L^{-1}, ZnSO$_4$·7H$_2$O 0.1 g L^{-1}, CuSO$_4$·5H$_2$O 0.0138 g L^{-1}. The inoculum of *A. niger* was added at a final count of 1×10^7 conidia (g dry CP)$^{-1}$. Moisture was adjusted to 80% (w v^{-1}) and packing density was 0.6 g mL^{-1}, measured by adding a known weight of wet substrate to an identified volume of the column. Fermentation was kept at 30 °C by circulating water inside a jacketed stainless steel cylindrical bioreactor, containing a helical tubing allowing for water circulation along the central axis of the cylinder [30]. A previous report from our group showed that fermentation time could be limited to 24 h [29]. In addition, according to the supplier CP contains residual starch, which might have accelerated fungal growth. Compressed air supply was sterilized using a Millex-FG$_{50}$ 5 cm filtration disk, with 0.2 μm pore size (Millipore, Bedford, MA, USA), bubbled through a 3 L flask containing sterile distilled water to produce nearly saturated air. Air flow was set at 0.2 vvm [(volume of air) (volume of substrate)$^{-1}$ (min)$^{-1}$]. Air passed through a stainless steel mesh located 5 cm above the bottom of the reactor and covered with 2 layers of cheese cloth. Aeration rate required by *A. niger* ranges 0.2–0.5 vvm for high enzyme productivity [31]. Higher air flow tended to dry the top section of the substrate in the reactor, while increasing moisture content at the bottom. The fermented product (5.5 kg) was mixed with 50 mM sodium acetate buffer, pH 5.5, using an equal weight ratio, and the mixture was stirred in an orbital shaker at 150 rpm, 4 °C, for 60 min. Subsequently, the liquid extract was separated and centrifuged at 10,000 × g for 30 min. The supernatant was filtered thought Whatman No. 1 filter paper to obtain a crude extract.

2.4. Scanning electron microscopy (SEM)

SEM was used to observe superficial morphological changes of CP before and after the alkaline pretreatment. Samples were dehydrated at 120 °C for 1 h, mounted on stubs and sputter-coated with gold/palladium for 300 s (EMS, model S550, Hatfield, PA, USA), using high vacuum and a voltage acceleration of 20 kV. SEM was performed in a Jeol JSM 5200 (Tokio, Japan) on previously coated CP.

2.5. Protein determination, β-xylosidase and xylanase enzymatic assays

Protein concentration was determined using the method of Bradford [32] with bovine serum albumin (BSA) as standard. Xylosidase activity was measured by the *p*-nitrophenol method [33], using 10 mM *p*-nitrophenol-β-D-xylopyranoside (PNPX) in 50 mM sodium acetate buffer, 30 μL of enzyme solution at pH 5.0 in a total reaction volume of 1.5 mL. After incubation at 60 °C for 10 min, the reaction was stopped by adding 1.0 M Na$_2$CO$_3$ (JT Baker, Phillipsburg, NJ, USA) to a final concentration of 0.3 mM, and the *p*-nitrophenol released was measured spectrophotometrically at 410 nm. One unit of β-xylosidase was considered as the amount of enzyme producing 1 μmol equivalent of *p*-nitrophenol per min. Xylanase activity was determined using birchwood xylan (5 g L^{-1}; Sigma), dissolved in 50 mM sodium acetate buffer, pH 5.5. Enzyme extracts (0.4 mL) were added to the substrate (0.4 mL) and incubated at 50 °C for 10 min followed by immersion in iced water. Reducing sugars released were quantified using the DNS method [34], and a xylose standard curve. One activity unit (U) was defined as the amount of enzyme that releases 1 μmol of xylose per min, at 50 °C.

2.6. Enzyme concentration and purification

The crude extract was concentrated by tangential flow ultrafiltration using a Pellicon unit (Millipore, Billerica, Massachusetts, USA), employing a 0.5 m^2 Biomax-5 (Millipore) polyethersulfone membrane of 5 kDa molecular weight cut off, at 4 °C. Tangential flux was 0.20 L m^{-2} h^{-1}, followed by dialysis against distilled water. Samples of the concentrated extract were partially purified by differential ammonium sulfate precipitation at 4 °C, using the following gradient: 0–25%, 25–50%, 50–80% and 80–100% saturation. After every step, samples were gently stirred for 1 h at 4 °C, centrifuged at 14,000 × g for 45 min, and supernatants were subjected to next precipitation stage and treated similarly. Precipitates were redissolved in 50 mM sodium acetate buffer, pH 5.5, dialyzed at 4 °C against the same buffer with three changes every 8 h, filtered through 0.45 μm membrane (Durapore, Millipore, Ireland) and tested for activity. The concentrated extract was partially purified using the best activity recovery conditions and was freeze dried (LabConCo, Freezone 18, Kansas City, MO, USA).

A 2.5 cm × 23 cm column filled with Macro-Prep High Q [binding capacity 40 mg protein (mL resin)$^{-1}$, Bio-Rad, Hercules, CA, USA] strong anion exchange, was pre-equilibrated with 50 mM sodium acetate buffer (pH 5.5). Ten mL (100 mg) protein sample was injected into the column. Elution was conducted using 80 mL of buffer at a flow rate of 1 mL min^{-1}, followed by 200 mL of a linear NaCl gradient (0–0.6 M) of same buffer, and a final elution using 200 mL of 0.6 M NaCl. Four mL fractions were collected, using a R-200 fraction collector (Pharmacia, Uppsala, Denmark). Active fractions were pooled, dialyzed and freeze dried, as previously mentioned.

Saturated ammonium sulfate was added to the anion exchange fraction containing β-xylosidase activity to 1.5 M final concentration. A sample of 10 mL (90 mg protein) was injected to a column (1.5 cm × 54 cm) filled with Macro-Prep methyl hydrophobic interaction chromatography [HIC, binding capacity >25 mg (mL resin)$^{-1}$, Bio-Rad], equilibrated with 20 mM sodium acetate buffer (pH 5.5) containing 1.5 M (NH$_4$)$_2$SO$_4$. The column was washed with 2-bed volumes of same buffer and eluted with a 7-bed volume decreasing (NH$_4$)$_2$SO$_4$ gradient (1.5–0 M) at flow rate of 1 mL min^{-1}. Four mL fractions were collected and active fractions were pooled, dialyzed and freeze dried.

2.7. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric point (pI)

Aliquots from pooled active fractions from HIC were subjected to SDS-PAGE using 10 T (% acrylamide plus bis-acrylamide in gelling solution) according to Laemmli [35] without β-mercaptoethanol, and heating at 60 °C for 5 min. Protein bands were stained with Coomassie brilliant blue G-250 (Bio-Rad). β-xylosidase activity was detected in the gel after electrophoresis by cutting the bands from non-stained gels. Each band was washed three times with Triton X-100 solution (2.5%, vv^{-1}), placed in a microtube containing 300 μL substrate solution (10 mM PNPX), and incubated for 60 min at 60 °C. The reaction was stopped by adding 300 μL of 1 M Na$_2$CO$_3$, and absorbance was measured at 400 nm, against a blank obtained by using a gel fragment without protein.

High molecular weight markers (GE Healthcare, Fairfield, CT, USA) were used to estimate the molecular weight of the purified enzyme. The isoelectric point of β-xylosidase was determined by using ultrathin (0.2 mm) polyacrylamide gels (5 T) for electrofocusing (100 mm × 125 mm) in a Bio-phoresis cell (Bio-Rad) system with IEF ampholites (3–10, Serva, Heidelberg, Germany). Protein bands were detected using silver staining [36].
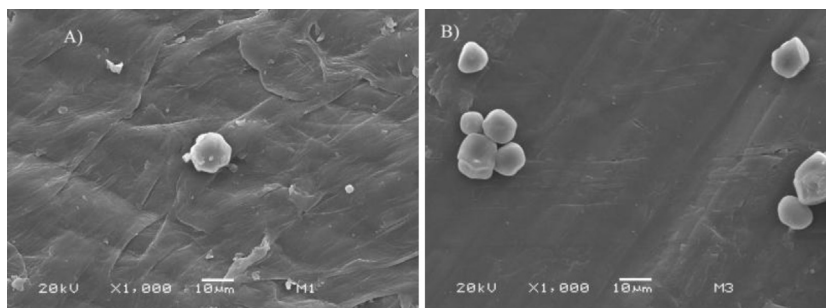
Fig. 1. Scanning electron microscopy (SEM) of CP surface. (A) untreated CP; (B) CP pretreated with AEW. Pretreated CP clearly shows degradation of a layer covering the hemicellulose fibers.

2.8. Kinetic properties and effect of temperature and pH on β-xylosidase activity

Concentrations of *p*-nitrophenyl-β-D-xylopyranoside were varied from 0.3 to 10 mM. Substrate dilutions were contacted with 0.2 μg β-xylosidase in sodium acetate buffer pH 5.0, at 60 °C, in a total volume of 300 μL. Kinetic parameters were estimated by fitting initial velocities (V_o) versus substrate concentrations to the Michaelis–Menten equation using a non-linear correlation curve-fitting software (Graph-Pad Prism 5.00; San Diego, CA, USA).

The effect of temperature on β-xylosidase activity, was evaluated between 40 °C and 75 °C, at optimal pH. The effect of pH was determined from 4.0 to 5.5 using 50 mM sodium acetate buffer, 50 mM sodium citrate buffer for pH 6.0, while 50 mM sodium phosphate buffer was used for pH between 6.5 and 7.5, at optimal temperature. Thermal stability was determined by incubating enzyme solutions in absence of substrate at 60 °C, 65 °C and 70 °C, taking samples every 10 min up to 1 h and immediately ice cooled. Residual activities were assayed under standard conditions.

2.8.1. Effect of chemicals on β-xylosidase activity

To evaluate the effect of various metal ions on β-xylosidase activity, 1 mM solutions of Fe^{2+}, Ca^{2+}, Cu^{2+}, Hg^{2+}, SDS, EDTA and 2-mercaptoethanol were added to the reaction mixtures containing the enzyme in 50 mM sodium acetate buffer (pH 5.0), at room temperature. A reaction mixture without any additive was used as control and its xylosidase activity was designated as 100%.

2.9. Far-UV circular dichroism (CD) spectra

The CD spectrum of purified β-xylosidase in 20 mM sodium acetate buffer pH 5.0, was recorded over the range 200–250 nm using a Jasco J-715 spectropolarimeter (Easton, MD, USA) fitted with a PTC-348WI Peltier-type cell holder. The 0.10 cm wide quartz cell was under constant nitrogen flush. Protein concentration (0.10 mg mL^{-1}) was determined by absorbance at 280 nm using an extinction coefficient of 217,165 M^{-1} cm^{-1}, calculated from a recombinant *A. niger* β-xylosidase [22], using the ProtParam tool of the ExPASy portal (http://web.expasy.org/protparam/). Native β-xylosidase spectra were obtained at 25 °C from freshly prepared samples, which were then heated to 90 °C and kept at this temperature until no further changes in ellipticity were noticed. CD spectra were then recorded for denatured β-xylosidase. Reversibility of thermal denaturation was tested by recording the spectra of these samples once cooled to 25 °C. Temperature-induced denaturation transitions were monitored by recording the ellipticity at 210 nm from 25 °C to 90 °C heating at a constant rate of 1.0 °C min^{-1}, using 1.0 cm cells. Renaturation profiles were recorded, once thermal denaturation was completed, by cooling to 25 °C at a rate of 1.0 °C min^{-1}. Ellipticities are reported as molar ellipticity [θ] (degrees cm^2 dmol^{-1}), based on an assumed mean amino acid residue weight (MRW) of 112 Da. Thus [θ] = θ (100 MRW) (c l)$^{-1}$, where c is protein concentration (mg mL^{-1}), l is light path length (cm), and θ is the measured ellipticity (degrees) [37]. CD spectra were reported as the mean of three scans corrected by subtracting the adequate blank runs on xylosidase-free buffer. CD data were employed to calculate secondary structure from the Dichroweb server (http://dichroweb.cryst.bbk.ac.uk) using the K2D method [38]. The thermal denaturation profile was analyzed by means of a two-state model, where the fraction of unfolded protein (f_U) is expressed as $f_U = K(1+K)^{-1}$, where the equilibrium constant is given by $K = \exp[-\Delta H(RT)^{-1} + \Delta S(R)^{-1}]$.

All data are expressed as mean ± SD of 3–5 experiments.

3. Results and discussion

3.1. Proximal analysis of CP

Hemicellulose was the major component of both CP pretreated with AEW and untreated CP, followed by cellulose, while lignin was relatively low (Table 1). The low lignin content allowed fungal growth on CP and thus SSF was used without previous treatment.

Table 1
CP proximal analysis of pretreated and untreated CP.

Chemical composition	Pretreated CP % (dry basis)	Untreated CP % (dry basis)
Moisture	8.75 ± 0.07*	8.15 ± 0.06*
Protein (Nx6.25)	5.54 ± 0.15	5.47 ± 0.03
Ether extract	0.36 ± 0.13	0.25 ± 0.03
Ash	0.90 ± 0.02	0.8 ± 0.03
Neutral detergent fiber (NDF)	83.27 ± 0.47	84.95 ± 0.77
Acid detergent fiber (ADF)	18.42 ± 0.21*	20.70 ± 0.23*
Hemicellulose	64.22 ± 0.64	64.25 ± 0.96
Cellulose	17.14 ± 0.21*	18.12 ± 0.23*
Lignin	1.28 ± 0.06*	2.58 ± 0.40*

Mean of three replicates ±standard deviation.
* Significant difference $p < 0.05$.

However, CP was treated with AEW in an attempt to enhance xylanolytic enzymes production.

3.2. Effect of AEW on CP

From SEM it was clear that AEW pretreatment modified the superficial structure of CP (Fig. 1). Initially CP showed an external layer probably comprising cutin and suberin (Fig. 1A), whereas after AEW pretreatment this layer almost disappeared and hemicellulose fibers were visible with thickness of about 5–6 μm (Fig. 1B). The evident surface exposure of these fibers represented a more available target for hemicellulolytic activity production during SSF. The width of hemicellulose fibers of Gingko (*Gingko biloba*) was about 3 ± 0.5 nm in a perpendicular plane orientation [39], while the diameter of raw CP fiber was reported as about 5 μm [40]. A study using distiller's dried grains with solubles (mainly corn fiber) pretreated with AEW reported disrupted crystalline structures and structural alterations, leading to increased amounts of fermentable sugars for biofuel production by microbial fermentation [41]. The amount of NaOH present in AEW confers surface active properties, enabling partial removal of waxes (long chain primary and secondary alcohols, aldehydes and ketones, and esterified fatty acids) associated to cutin and suberin of CP [39,42,43]. However, the difference between lipid content (ether extract) of AEW pretreated and untreated CP was not significant (Table 1), suggesting another more significant effect of AEW pretreatment. Alkali pretreatment [2%, w v^{-1}, Ca(OH)$_2$] was reported to remove the waxy cutin layer of CP leading to exposure of hemicelluloses fibers [40].

Partial delignification of CP cell wall was observed after AEW pretreatment, and this was confirmed by ADF determination, that evaluates the sum of cellulose plus lignin which resulted in significantly lower value for AEW pretreated than untreated CP (Table 1). This effect probably allowed an easier fungal accessibility to the substrate upon SSF. AEW pretreatment represents an
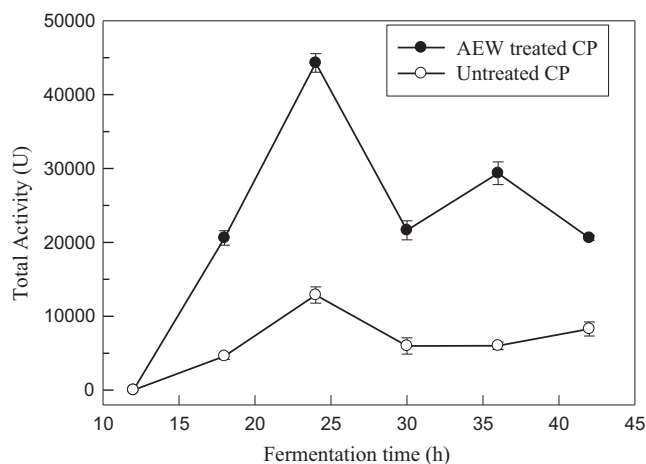
Fig. 2. Comparison of the total xylanase activity produced after fermentation of pretreated CP with AEW diluted 1:4, contact time of 5 h at room temperature ($25 \pm 2\,^\circ$C). Error bars indicate standard deviation of the mean of three replicates.

Fig. 3. Macro-Prep high Q anionic exchange chromatography of β-xylosidase from *Aspergillus niger* GS1. The active fractions were eluted with a linear gradient of NaCl from 0 to 0.6 M.

environmentally friendly alternative to the use of hazardous chemical pretreatments, which then require appropriate disposal.

The basic structure of CP arabinoxylans consists of (1–4)-β-D-xylopyranosyl (Xylp) backbone, with α-L-arabinofuranosyl (Araf) side units attached to O-2 and/or O-3 of backbone Xylp units. Additionally, ferulic acid can be esterified to the arabinose side-chains and create cross-links with other arabinoxylan chains [44,45]. AEW pretreatment may have produced partial diferulates hydrolysis leading to better fungal accessibility during SSF in relation to higher xylanolytic enzymes production, with essentially no change in hemicellulose content of AEW pretreated and untreated CP (Table 1).

3.3. SSF of AEW pretreated CP

Total xylanase activity production by *A. niger* on AEW pretreated CP after 24 h of fermentation was about 3.4 fold over the untreated substrate. Adequate nutrients supplementation combined with available carbon sources allowed a rapid fungal growth whereas xylosidase activity could not be detected (Fig. 2). In a similar study β-xylosidase could not be detected in the crude extract [46]. The protein solution (0.09 mg mL^{-1}) was concentrated by ultrafiltration from 5.5 L to 200 mL. Activity recoveries from concentrated crude extract using differential ammonium sulfate precipitation were: 5.7% for 0–25% fraction, 6.4% for 25–50% fraction, 64% for 50–80% fraction and 2.6% for 80–100% fraction. After using the 50–80% fraction, the final extract was dialyzed achieving a volume of 80 mL showing a protein concentration of 1.4 mg mL^{-1}, and β-xylosidase activity of 0.30 U (mg protein)$^{-1}$.

3.4. Purification of β-xylosidase

Xylanolytic activity was stable at room temperature for several hours and therefore all purification steps were performed at
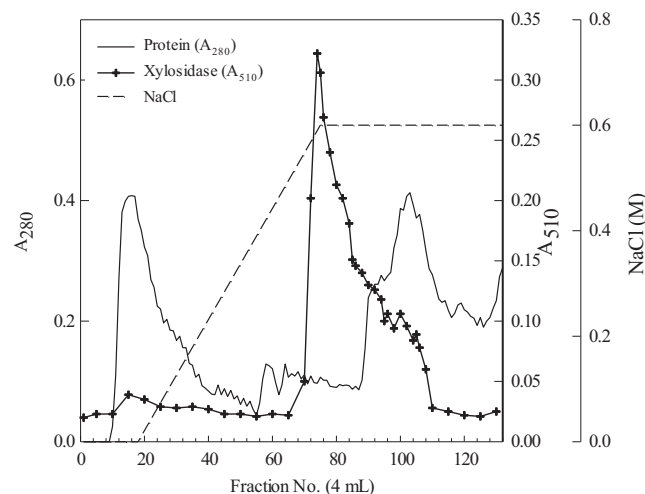
this temperature. From anion exchange chromatography xylanase activity was separated in fractions 15–24, giving an activity of 21 U mg^{-1}. β-xylosidase activity was detected in fractions 70–82 eluted at 0.58–0.6 M NaCl gradient (Fig. 3). These fractions contained 14% of total injected protein and showed a specific activity of 9.8 U (mg protein)$^{-1}$, which were pooled, dialyzed and freeze dried. After HIC β-xylosidase activity was detected in 10 fractions (results not shown), which were pooled and dialyzed giving a specific activity of 386.7 U (mg protein)$^{-1}$. A summary of the purification steps required to achieve β-xylosidase is shown in Table 2.

3.4.1. Biochemical and kinetic properties of β-xylosidase

The optimal temperature of β-xylosidase under assay conditions was 65 °C (Fig. 4A), which was lower than that of native and recombinant β-xylosidase from *Aspergillus niger* [22,47]. In addition, the enzyme showed activity at acidic pH only with an optimum pH of 4.5, and $\geq$70% activity was observed from pH 3.5 to 6.0 (Fig. 4B). These values were similar to other β-xylosidases isolated from *Aspergillus carbonarius* [2]. Good thermal stability results were found for β-xylosidase because it was fully stable at 60 °C for 60 min (Fig. 4C). Incubation at 65 °C resulted in 70% activity retention after 30 min, whereas at 70 °C activity retained was 10% at same incubation time. Furthermore, 20% relative activity was retained after only 10 min of incubation at 70 °C (Fig. 4C). β-xylosidase produced by *A. niger* USP-67 was purified and immobilized in polyethyleneimine-sepharose; it was more thermal stable than the soluble enzyme and on other supports, and showed 50% activity loss after 50 min, at 65 °C [48]. Thus, thermal stability was slightly lower than our purified β-xylosidase (62% activity retention after 50 min at 65 °C, Fig. 4C). On the other hand, *A. niger* IBT-3250 was more thermally stable, showing 44% activity retention after 1 h at 75 °C [33].

From SDS-PAGE purified β-xylosidase showed a molecular weight (MW) of 111 kDa (Fig. 5A) under reducing and non

Table 2
Summary of the purification procedure to obtain β-xylosidase from *A. niger* GS1.

Purification steps	Protein (mg)	Total activity (U)	Specific activity [U (mg protein)$^{-1}$]	Yield (%)	Purification (fold)
Crude extract	4987	1514	0.30	100	1.00
(NH$_4$)$_2$SO$_4$ precipitation and concentration	114.1	156.4	1.37	10.3	4.57
AEC[a]	15.7	153.9	9.80	10.2	32.67
HIC[b]	0.019	7.28	386.7	0.48	1289

[a] Macro-Prep High Q anion exchange chromatography (Bio-Rad).
[b] Macro-Prep methyl hydrophobic interaction chromatography (Bio-Rad).
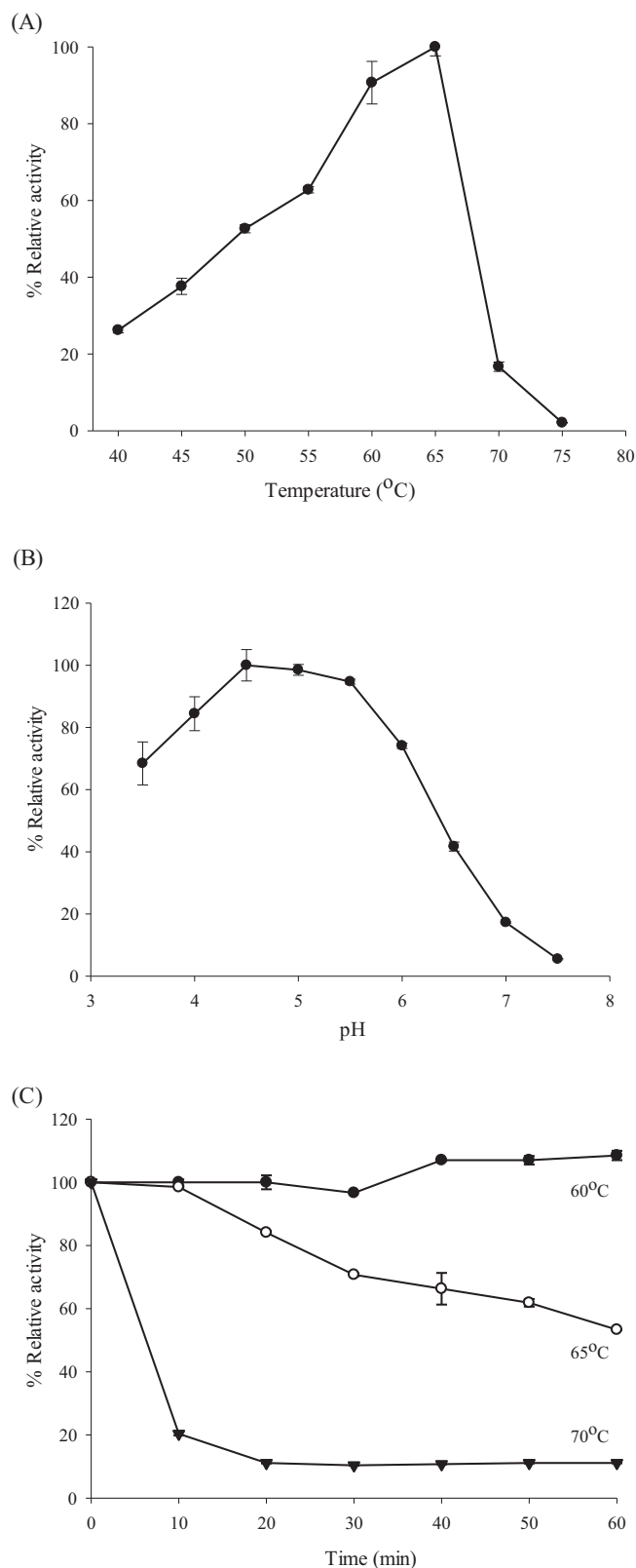
(A)

(B)

(C)

Fig. 4. Effect of pH and temperature on β-xylosidase activity. (A) Effect of pH at 25 °C. Activity was measured at 60 °C for 15 min at the indicated pH. (B) Effect of temperature. Activity was measured at pH 5.0 for 15 min at the indicated temperature. (C) Effect of temperature on β-xylosidase stability. Ordinate values are expressed as relative activity, i.e. ratio of actual activity divided by maximum activity expressed as percentage. Results represent the mean of three experiments, and bars represent standard deviation.
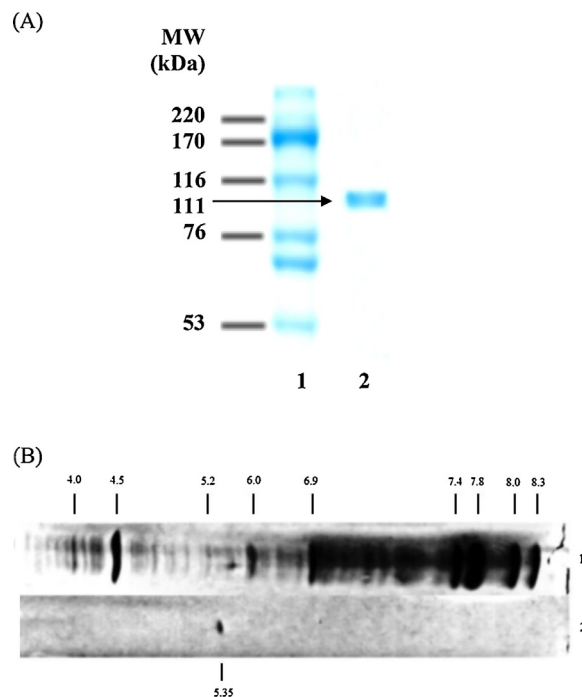
(A)

(B)

Fig. 5. (A) SDS-PAGE of purified β-xylosidase from *Aspergillus niger* GS1. Lane 1, molecular weight markers: myosin rabbit muscle (220 kDa), 2-macroglobulin bovine plasma (170 kDa), galactosidase from *E. coli* (116 kDa), human transferrin (76 kDa), bovine liver glutamic dehydrogenase (53 kDa). Lane 2, purified protein. (B) Isoelectric focusing. Lanes: 1 IEF ampholites (range 3-10); 2, purified protein. Bands were detected by silver staining.

reducing conditions, suggesting a monomeric protein (results not shown). Most fungal β-xylosidases exhibit MW above 100 kDa [20], whereas those produced by *Aspergillus* sp. range 60–190 kDa [10], suggesting that our enzyme size is expected for this type of fungi. From isoelectric focusing purified β-xylosidase showed a p*I* of 5.35, indicating acidic nature of the enzyme (Fig. 5B). We could only find one report on the p*I* of β-xylosidase from *Aspergillus* and corresponds to *A. versicolor* with a value of 5.6 [49], all other reported β-xylosidases show p*I* values above and below that found in this study, but remain within the values reported for other filamentous fungi (3.4–7.8) [10].

From non-linear correlation curve using the Graph-Pad Prism software, the K_m of β-xylosidase was 6.1 ± 0.9 mM, V_{max} was 1364 ± 105 U (mg protein)$^{-1}$, while the k_{cat} value was 5.1 ± 0.1 s^{-1}. The apparent second order rate constant [k_{cat} (K_M)$^{-1}$] was 0.84 s^{-1} mM^{-1}. The K_M value obtained here was higher than other reported for recombinant (0.48 mM) [50] and native β-xylosidases (0.25 ± 0.07 mM) [51], (0.17–0.22 mM) [2] and 0.66 mM [52], suggesting lower substrate affinity. In this study V_{max} was 35 times higher than that reported for β-xylosidase from *A. ochraceus* [52] implicating higher hydrolysis rate for same substrate concentration. The k_{cat} value was smaller than that reported for β-xylosidase from *Aspergillus awamori* X-100 (17.5 ± 2.0 s^{-1}) [51], indicating one third catalytic efficiency. More studies are necessary to fully evaluate hydrolytic specificity and analysis of hydrolysis products.

3.4.2. Influence of chemicals on activity of β-xylosidase

The activity of β-xylosidase was measured in the presence of metal ions and other agents (Table 3). The enzyme was not affected by 1.0 mM Fe^{2+}, Ca^{2+}, Cu^{2+} and EDTA. The fact that EDTA did not affect activity, suggests that no metal cofactors are needed for the enzymatic reaction, whereas Hg^{2+} showed a partial inhibition. β-xylosidase was markedly inactivated by β-mercaptoethanol indicating that disulfide bridges have a significant effect on protein

Table 3
Influence of various chemicals on activity of β-xylosidase from *A. niger* GS1.

Chemical (1 mM)	% Relative activity
None	100
SDS	111
EDTA	103
β-Mercaptoethanol	54
FeSO$_4$	98
CaCl$_2$	101
CuSO$_4$	105
HgCl$_2$	89

active conformation. Slight enzyme inhibition was found in studies where SDS was tested [22,53]. However, this detergent caused a slight activity increase to our xylosidase, perhaps because the active site was more exposed to the substrate than in native conformation.

3.5. CD studies on β-xylosidase

The far-UV CD spectrum of native β-xylosidase (Fig. 6) is characteristic of proteins showing α/β structure [7], where the broad minimum around 210 nm and the shoulder close to 220 nm suggest α-helix together with β-sheet conformations [54]. Deconvolution of CD spectra showed 41% α-helix, 16% β-sheet and 43% random (root mean square deviation = 0.125). Secondary structure of *Trichoderma reesei* β-xylosidase showed little similarity to our results (23% α-helix, 27% β-sheet) [7], indicating that our enzyme probably belongs to another GH family, which might be assigned after N-terminal sequencing. β-xylosidase from *Geobacillus pallidus* [37] showed a high percentage of α-helix (44%) in agreement with our results, but a higher amount of β-sheet (40%), probably influenced by the use of a neutral pH, while our study was conducted at the optimum pH (5.0). On the other hand, unlike our results the same α-helix and β-sheet content (30%) were found for *Geobacillus stearothermophilus* xylosidase at physiological pH [54]. The loss of secondary structure of β-xylosidase kept at 90 °C is evidenced by the shoulder shown between 210 and 220 nm and the negative band at about 203 nm, both of which associated with thermally unfolded proteins (Fig. 6) [55]. However, under the gradual cooling used here a large part of the native secondary structure was recovered, indicating that good reversibility was achieved after thermal denaturation. Indeed, the CD spectrum of the protein cooled back to 25.0 °C was consistent with the presence of 37% helix, 16% β-sheet, and 48% random conformation. Analysis of thermal transition profiles indicated melting temperature (Tm, midpoint of the transition from α/β to random structure) of 54.1 ± 5.8 °C; the calculated enthalpy change for unfolding was 67.4 ± 6.7 kJ (mol)$^{-1}$,

which is slightly higher than that reported for a *Kluyveromices marxianus* xylosidase [56]. Onset temperature (To, where the loss of ellipticity signal reaches 5% of the original) for β-xylosidase was 37 °C, which indicates a relatively good initial stability.

4. Conclusion

Pretreatment of CP with AEW increased total xylanase activity production by 3.4 fold after solid state fermentation and a new β-xylosidase from *Aspergillus niger* GS1 was identified and purified. Biochemical, thermodynamic and structural properties of the enzyme are different to those reported previously. Further studies are needed to assess its applications in industrial processes such as fruit juice clarification, enzymatic conversion of xylooligosaccharides into monosaccharides and baking industry.
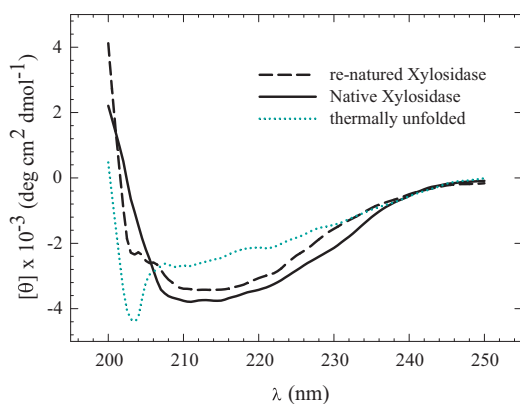
Acknowledgments

Fig. 6. Far-UV CD spectra of β-xylosidase: native (25 °C), thermally unfolded (evaluated at 90 °C), and re-natured enzyme (25 °C). Spectra were recorded in 20 mM sodium acetate buffer (pH 5.0), at 0.10 mg mL^{-1} in a 0.1 cm cell.

References

[1] Bastawde K. Xylan structure, microbial xylanases, and their mode of action. World J Microbiol Biotechnol 1992;8(4):353–68, http://dx.doi.org/10.1007/BF01198746.

[2] Kiss T, Erdei A, Kiss L. Investigation of the active site of the extracellular β-xylosidase from *Aspergillus carbonarius*. Arch Biochem Biophys 2002;399(2):188–94, http://dx.doi.org/10.1006/abbi.2002.2753.

[3] Saha B. Hemicellulose bioconversion. J Ind Microbiol Biotechnol 2003;30(5):279–91, http://dx.doi.org/10.1007/s10295-003-0049-x.

[4] Watson SA, Ramstad PE. Corn: chemistry and technology. St. Paul, Minnesota: AACC; 1987. p. 53–82.

[5] van Wyk JPH. Biotechnology and the utilization of biowaste as a resource for bioproduct development. Trends Biotechnol 2001;19(5):172–7, http://dx.doi.org/10.1007/s00449-012-0705-5.

[6] Facchini FDA, Vici AC, Benassi VM, Freitas LAP, Reis RA, Jorge JA, Terenzi HF, Polizeli MLTM. Optimization of fibrolytic enzyme production by *Aspergillus japonicus* C03 with potential application in ruminant feed and their effects on tropical forages hydrolysis. Bioprocess Biosyst 2011;34:1027–38, http://dx.doi.org/10.1007/s00449-011-0553-8.

[7] Rojas AL, Fischer H, Eneiskaya EV, Kulminskaya AA, Shabalin KA, Neustroev KN, Craievich AF, Golubev AM, Polikarpov I. Structural insights into the β-xylosidase from *Trichoderma reesei* obtained by synchrotron small-angle X-ray scattering and circular dichroism spectroscopy. Biochemistry 2005;44(47):15578–84, http://dx.doi.org/10.1021/bi050826j.

[8] Wong K, Tan L, Saddler J. Multiplicity of β-1,4-xylanase in microorganisms: functions and applications. Microbiol Rev 1988;52(3):305–17.

[9] Biely P. Xylanolytic enzymes. In: Whitaker J, Dominic WS, editors. Handbook of food enzymology. New York: CRC Press; 2002. p. 879–915, http://dx.doi.org/10.1201/9780203910450 [Chapter 71].

[10] Knob A, Terrasan CRF, Carmona EC. β-Xylosidases from filamentous fungi: an overview. World J Microbiol Biotechnol 2010;26(3):389–407, http://dx.doi.org/10.1007/s11274-009-0190-4.

[11] Sunna A, Antranikian G. Xylanolytic enzymes from fungi and bacteria. Crit Rev Biotechnol 1997;17(1):39–67, http://dx.doi.org/10.3109/07388559709146606.

[12] Pal A, Khanum F. Production and extraction optimization of xylanase from *Aspergillus niger* DFR-5 through solid-state-fermentation. Bioresour Technol 2010;101(19):7563–9, http://dx.doi.org/10.1016/j.biortech.2010.04.033.

[13] Singhania RR, Sukumaran RK, Patel AK, Larroche C, Pandey A. Advancement and comparative profiles in the production technologies using solid-state and submerged fermentation for microbial cellulases. Enzyme Microb Technol 2010;46(7):541–9, http://dx.doi.org/10.1016/j.enzmictec.2010.03.010.

[14] Pandey A. Solid-state fermentation. Biochem Eng J 2003;13(2–3):81–4, http://dx.doi.org/10.1016/S1369-703X(02)00121-3.

[15] Pei H, Liu L, Zhang X, Sun J. Flow-through pretreatment with strongly acidic electrolyzed water for hemicellulose removal and enzymatic hydrolysis of corn stover. Bioresour Technol 2012;110(April):292–6, http://dx.doi.org/10.1016/j.biortech.2011.12.062.

[16] Wang X, Feng H, Li Z. Pretreatment of switchgrass with electrolyzed water and a two-stage method for bioethanol production. Biotechnol Bioprocess Eng 2012;17(3):624–33, http://dx.doi.org/10.1007/s12257-011-0583-8.

[17] Wang X, Feng H, Li ZY. Ethanol production from corn stover pretreated by electrolyzed water and a two-step pretreatment method. Chin Sci Bull 2012;57(15):1796–802, http://dx.doi.org/10.1007/s11434-012-5079-1.

[18] Wang X, Wang Y, Wang B, Blaschek H, Feng H, Li Z. Biobutanol production from fiber-enhanced DDGS pretreated with electrolyzed water. Renew Energy 2013;52(April):16–22, http://dx.doi.org/10.1016/j.renene.2012.10.011.

[19] Pei H, Sun J, Liu L, Hu J, Zhang X, Zhang J. Pretreatment of corn stover by acidic electrolyzed water for enhancing hemicellulose degradation. J Biobased Mater Bioenergy 2011;5(3):403–8, http://dx.doi.org/10.1166/jbmb.2011.1164.

[20] Romero AM, Mateo JJ, Maicas S. Characterization of an ethanol-tolerant 1,4-B-D-xylosidase produced by *Pichia membranifaciens*. Lett Appl Microbiol 2012;55(5):354–61, http://dx.doi.org/10.1111/j.1472-765X.2012.03297.x.

[21] Glycoside hydrolase. Family 30; 2012. http://www.cazy.org/GH30.html [accessed 31.08.2012].

[22] Amaro-Reyes A, García-Almendárez B, Vázquez-Mandujano D, Amaya-Llano S, Castaño-Tostado E, Guevara-González R, Loera O, Regalado C. Homologue expression of a β-xylosidase from native *Aspergillus niger*. J Ind Microbiol Biotechnol 2010;38(9):1311–9, http://dx.doi.org/10.1007/s10295-010-0912-5.

[23] AOAC ed. Method 930.15. Loss on drying (moisture) for feeds. In: Horwitz W, editor. Official methods of analysis of AOAC international, vol. I, 17th ed. Maryland, USA: AOAC International; 2002.

[24] AOAC ed. Method 954.01. Protein (crude) in animal feed and pet food. In: Horwitz W, editor. Official methods of analysis of AOAC international, vol. I, 17th ed. Maryland, USA: AOAC International; 2002.

[25] AOAC, Method 920.39. Fat (crude) or ether extract in animal feed. In: Horwitz W, editor. Official methods of analysis of AOAC international, vol. I, 17th ed. Maryland, USA: AOAC International; 2002.

[26] AOAC, ed. Method 942.05. Ash of animal feed. In: Horwitz W, editor. Official methods of analysis of AOAC international, vol. I., 17th ed. USA: AOAC International: Maryland; 2002.

[27] Ankom T. Neutral detergent fiber in feeds, filter bag technique, Method 6. NY: Macedon; 2011.

[28] ANKOM T. Acid detergent fiber in feeds, filter bag technique, method 5. NY: Macedon; 2011.

[29] Regalado C, Vázquez-Obregón I, García-Almendárez BE, Domínguez-Domínguez J, Aguilera-Barreyro A, Amaro-Reyes A. Xylanolytic enzymes production by *Aspergillus niger* GS1 from solid-state fermentation on corn stover and their effect on ruminal digestibility. Elect J Biotechnol 2011;14(2), http://dx.doi.org/10.2225/vol14-issue2-fulltext-3.

[30] Lagunas-Bernabé I, Garcia-Almendárez B, Castaño-Tostado E, Regalado González C, Ávila González E. Production of hemicellulolytic enzymes through solid substrate fermentation and their application in the production of balanced feed for broilers. Vet Mex 2006;37(1):1–13.

[31] Garcia-Kirchner O, Segura-Granados M, Rodriguez-Pascual P. Effect of media composition and growth conditions on production of β-glucosidase by *Aspergillus niger* C-6. Appl Biochem Biotechnol 2005;121(1–3):347–59.

[32] Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 1976;72(1–2):248–54, http://dx.doi.org/10.1016/0003-2697(76)90527-3.

[33] Pedersen M, Lauritzen H, Frisvad J, Meyer A. Identification of thermostable β-xylosidase activities produced by *Aspergillus brasiliensis* and *Aspergillus niger*. Biotechnol Lett 2007;29(5):743–8, http://dx.doi.org/10.1007/s10529-007-9314-9.

[34] Miller GL. Use of dinitrosalicilyc acid reagent for determination of reducing sugar. Anal Chem 1959;31(3):426–8.

[35] Laemmli U. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227(5259):680–5, http://dx.doi.org/10.1038/227680a0.

[36] Blum H, Beier H, Gross HJ. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis 1987;8(2):93–9, http://dx.doi.org/10.1002/elps.1150080203.

[37] Quintero D, Velasco Z, Hurtado-Gomez E, Neira JL, Contreras LM. Isolation and characterization of a thermostable β-xylosidase in the thermophilic bacterium *Geobacillus pallidus*. BBA-Proteins Proteom 2007;1774(4):510–8, http://dx.doi.org/10.1016/j.bbapap.2007.02.002.

[38] St John FJ, González JM, Pozharski E. Consolidation of glycosyl hydrolase family 30: A dual domain 4/7 hydrolase family consisting of two structurally distinct groups. FEBS Lett 2010;584(21):4435–41, http://dx.doi.org/10.1016/j.febslet.2010.09.051.

[39] Terashima N, Kitano K, Kojima M, Yoshida M, Yamamoto H, Westermark U. Nanostructural assembly of cellulose, hemicellulose, and lignin in the middle layer of secondary wall of ginkgo tracheid. J Wood Sci 2009;55(6):409–16, http://dx.doi.org/10.1007/s10086-009-1049-x.

[40] Briones FC, Iribarren A, Peña J, Rodríguez RC, Oliva A. Recent advances on the understanding of the nixtamalization process. Superficies y vacío 2000;10:20–4.

[41] Wang B, Ezeji T, Shi Z, Feng H, Blaschek HP. Pretreatment and conversion of distiller's dried grains with solubles for acetone–butanol–ethanol (ABE) production. Trans ASABE 2009;52(3):885–92.

[42] Bunzel M, Schüßler A, Saha G. Chemical characterization of Klason lignin preparations from plant-based foods. J Agric Food Chem 2011;59(23):12506–13, http://dx.doi.org/10.1021/jf2031378.

[43] Rowland O, Domergue F. Plant fatty acyl reductases: enzymes generating fatty alcohols for protective layers with potential for industrial applications. Plant Sci 2012;193-194:28–38, http://dx.doi.org/10.1016/j.plantsci.2012.05.002.

[44] Bunzel M, Ralph J, Marita JM, Hatfield RD, Steinhart H. Diferulates as structural components in soluble and insoluble cereal dietary fibre. J Sci Food Agric 2001;81:653–60, http://dx.doi.org/10.1021/jf2031378.

[45] Shallom D, Shoham Y. Microbial hemicellulases. Curr Opin Microbiol 2003;6(3):219–28, http://dx.doi.org/10.1016/S1369-5274(03)00056-0.

[46] Semenova M, Drachevskaya M, Sinitsyna O, Gusakov A, Sinitsyn A. Isolation and properties of extracellular β-xylosidases from fungi *Aspergillus japonicus* and *Trichoderma reesei*. Biochem (Moscow) 2009;74(9):1002–8, http://dx.doi.org/10.1134/S0006297909090089.

[47] Delcheva G, Dobrev G, Pishtiyski I. Performance of *Aspergillus niger* B 03 β-xylosidase immobilized on polyamide membrane support. J Mol Catal B: Enzym 2008;54(3–4):109–15, http://dx.doi.org/10.1016/j.molcatb.2007.12.019.

[48] Benassi VM, Marcio-da Silva T, Pessela BC, Guisan JM, Mateo C, Lima MS, Jorge JA, Polizeli MLTM. Immobilization and biochemical properties of a β-xylosidase activated by glucose/xylose from *Aspergillus niger* USP-67 with transxylosylation activity. J Mol Catal B: Enzym 2013;89:93–101, http://dx.doi.org/10.1016/j.molcatb.2012.12.010.

[49] Andrade S, Polizeli MLTM, Terenzi HF, Jorge JA. Effect of carbon source on the biochemical properties of β-xylosidases produced by *Aspergillus versicolor*. Process Biochem 2004;39(12):1931–8, http://dx.doi.org/10.1016/j.procbio.2003.09.024.

[50] Suzuki S, Fukuoka M, Ookuchi H, Sano M, Ozeki K, Nagaioshi E, Takii Y, Matsushita M, Tada S, Kusumoto KI. Characterization of *Aspergillus oryzae* glycoside hydrolase family 43 β-xylosidase expressed in *Escherichia coli*. J Biosci Bioeng 2010;109(2):115–7, http://dx.doi.org/10.1016/j.jbiosc.2009.07.018.

[51] Eneyskaya EV, Ivanen DR, Bobrov KS, Isaeva-Ivanova LS, Shabalin KA, Savel'ev AN, Golubev AM, Kulminskaya AA. Biochemical and kinetic analysis of the GH3 family β-xylosidase from *Aspergillus awamori* X-100. Arch Biochem Biophys 2007;457(2):225–34, http://dx.doi.org/10.1016/j.abb.2006.10.024.

[52] Michelin M, Meixoto-Nogueira SC, Silva TM, Jorge JA, Terenzi HF, Teixeira JA, Polizeli MLTM. A novel xylan degrading β-D-xylosidase: purification and biochemical characterization. World J Microb Biotechnol 2012;28(11):3179–86, http://dx.doi.org/10.1007/s11274-012-1128-9.

[53] Kumar S, Ramón D. Purification and regulation of the synthesis of β-xylosidase from *Aspergillus nidulans*. FEMS Microbiol Lett 1996;135(2–3):287–93, http://dx.doi.org/10.1111/j.1574-6968.1996.tb08003.x.

[54] Contreras L, Gómez J, Prieto J, Clemente-Jiménez J, Las Heras-Vázquez F, Rodríguez-Vico F, Blanco F, Neira J. The family 52 β-xylosidase from *Geobacillus stearothermophilus* is a dimer: structural and biophysical characterization of a glycoside hydrolase. BBA-Proteins Proteom 2008;1784(12):1924–34, http://dx.doi.org/10.1016/j.bbapap.2008.06.019.

[55] Toledo-Núñez C, López-Cruz JI, Hernández-Arana A. Thermal denaturation of a blue-copper laccase: formation of a compact denatured state with residual structure linked to pH changes in the region of histidine protonation. Biophys Chem 2012;167:36–42, http://dx.doi.org/10.1016/j.bpc.2012.04.004.

[56] Rajoka MI. Kinetic parameters and thermodynamic values of β-xylosidase production by *Kluyveromyces marxianus*. Bioresour Technol 2007;98(11):2212–9, http://dx.doi.org/10.1016/j.biortech.2006.08.029.