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Characterization and biotechnological application of recombinant xylanases from *Aspergillus nidulans*

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ABSTRACT

Two xylanases from *Aspergillus nidulans*, XlnB and XlnC, were expressed in *Pichia pastoris*, purified and characterized. XlnB and XlnC achieved maximal activities at 60 °C and pH 7.5 and at 50 °C and pH 6.0, respectively. XlnB showed to be very thermostable by maintaining 50% of its original activity after 49 h incubated at 50 °C. XlnB had its highest activity against wheat arabinoxylan while XlnC had the best activity against beechwood xylan. Both enzymes were completely inhibited by SDS and $HgCl_2$. Xylotriose at 1 mg/ml also totally inibited XlnB activity. TLC analysis showed that the main product of beechwood xylan hydrolysis by XlnB and XlnC was xylotetraose. An additive effect was shown between XlnB and XlnC and the xylanases of two tested commercial cocktails. Sugarcane bagasse saccharification results showed that these two commercial enzymatic cocktails were able to release more glucose and xylose after supplementation with XlnB and XlnC.

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1. Introduction

Hemicellulose is the second most abundant organic material in the world and it presents a very complex structure composed of various residues [1,2]. Xylan, the major hemicellulose polymer, is hydrolyzed by β-1,4-endoxylanase, β-1,4-xylosidase and accessory enzymes [3–5]. β-1,4-*E*ndoxylanases (E.C. 3.2.1.8) are glycoside hydrolases that catalyze the hydrolysis of β-1,4-xylosidic linkages of the xylan backbone [6]. They are produced by many organisms but their main commercial sources are a small number of filamentous ascomycete fungi [7].

β-1,4-*E*ndoxylanases from fungi belong to two glycoside hydrolase families: GH10 and GH11, and these two families differ in their substrate specificity [7,8]. Both families catalyze the hydrolysis of xylan through the retention of the anomeric configuration, and two glutamate residues have been implicated in their catalytic mechanism [9]. However, GH10 endoxylanases have a broader substrate specificity and are therefore very important for complete degradation of substituted xylans [8,10]. They also differ with respect to their catalytic domains, which in GH10 endoxylanases present an α/β8 TIM-barrel topology, while GH11endoxylanases have a jelly-roll structure [2,11].

Xylanases can be used for various biotechnological applications such as bioconversion of lignocellulose into fermentable sugars, clarification of juices, preparation of animal feed and pulp bleaching [12,13]. However, for industrial applications, xylanases need to have desirable properties such as stability in a wide pH and temperature range, high specific activities and low production costs [14,15]. Therefore, molecular techniques have been used to obtain xylanases with ideal characteristics for commercial purposes [7].

Heterologous expression in yeast has a lot of advantages among which the production of soluble and correctly folded recombinant proteins that have undergone post-translational modifications, the capacity of growth at high cell densities and the ability to secrete reasonable amounts of protein [16]. *Pichia pastoris* has become a well described and widely applied expression host for xylanase production mainly because this yeast gives high expression under its own promoters [17]. Furthermore, there is no secretion of endogenous lignocellulolitic enzymes in significant amounts by *P. pastoris* and the recombinant proteins are almost pure heterologous enzyme preparations [18]. In addition, proteins can be obtained by inexpensive large-scale cultivation of *P. pastoris* which reduce costs of the process [19].

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Two xylanases from *Aspergillus nidulans* were purified and characterized [20–22] and the genes which express these xylanases, AN1818 (*xlnB*) and AN3613 (*xlnC*), were cloned in *P. pastoris* [23,24]. Although the natively produced enzymes from *A. nidulans* were previously studied, the functional properties of these heterologous xylanases and their potential for lignocellulosic biomass hydrolysis remained largely unexplored.

In this work, *xlnB* and *xlnC* were produced by *P. pastoris* and the corresponding xylanases, XlnB (GH10) and XlnC (GH11), respectively, were purified and biochemically characterized. Their ability to supplement commercial enzymatic cocktails for use in sugarcane bagasse hydrolysis was evaluated.

2. Materials and methods

2.1. Materials

Substrates including *p*-nitrophenyl-β-D-glucopyranoside (pNPβGlc), *p*-nitrophenyl-β-D-xylopyranoside (pNPβXyl), ρ-nitrophenyl-β-D-mannopyranoside (pNPβMan), ρ-nitrophenyl-α-D-mannopyranoside (pNPαMan), *p*-nitrophenyl-β-D-galactopyranoside (pNPβGal), *p*-nitrophenyl-α-D-galactopyranoside (pNPαGal), *p*-nitrophenyl-α-D-arabinofuranoside (pNPαAra), *p*-nitrophenyl-β-D-cellobioside (pNPβCel), carboxymethylcellulose (CMC), Avicel®, xylan from birchwood, xylan from beechwood, oat spelt xylan, dinitrosalicylic acid (DNS) and methanol were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Arabinoxylan (wheat flour, low viscosity – 8 cSt) was obtained from Megazyme (Wicklow, Ireland). Yeast extract was purchased from Himedia Laboratories Co. (Mumbai, Maharashtra, India). The chemical reagents NaOH, H_2SO_4 and potassium sodium tartrate were obtained from Vetec Fine Chemical (Duque de Caxias, RJ, Brazil). The commercial enzymatic mixtures Multifect® CL and Accellerase® 1500 were purchased from Genencor International Inc. (Rochester, NY, USA). Sugarcane bagasse was kindly donated by Jatiboca Sugar and Ethanol Plant, Urucânia, MG, Brazil. All others reagents used in this study were of analytical grade.

2.2. Pichia pastoris *strains and cultivation conditions*

The *P. pastoris* strains used in this study were previously described [23,24]. The strains were grown in 25 ml of buffered complex glycerol medium (BMGY) in 250 ml shake flasks at 28 °C and 250 rpm for 16 h. Aliquot cells were diluted to an OD 1.0 (600 nm) with 25 ml of buffered complex methanol medium (BMMY) and incubated for 72 h with daily addition of 150 μl of 100% methanol. The culture was centrifuged at 5000 rpm for 10 min according to the Pichia Expression Kit Manual (Invitrogen).

2.3. Protein analysis

Protein concentration in the enzymatic extract of *P. pastoris* and in the commercial enzymatic mixtures was determined by the Coomassie Blue binding method using bovine serum albumin (BSA) as a standard [25].

2.4. Xylanase assay

All enzymatic assays were carried out in 100 mM sodium acetate buffer, pH 5, at 50 °C. They were performed in triplicate and the mean values were calculated. Relative standard deviations of the measurements were below 5%. Xylanase activity was determined using xylan from beechwood (1% w/v at final concentration) as substrate. The enzymatic reactions were initiated by the addition of 100 μl of the appropriately diluted enzyme solution to 400 μl of the polysaccharide substrate solution prepared in buffer. The reaction mixtures were incubated for 15 min and the total reducing sugar content released was determined at 540 nm by DNS method [26] using xylose as standard. One enzyme unit (U) was defined as the amount of enzyme necessary to produce 1 μmol of xylose equivalent per minute.

2.5. Xylanase purification

The crude extracts from *P. pastoris* were centrifuged at 15,000*g* for 30 min at 4 °C. The histidine tags were used for the purification of the recombinant proteins by nickel affinity chromatography. The xylanase purification was carried out at room temperature under native condition, according to protocols described in The QIAExpressionist™ Mannual (Fifth Edition, March 2001).

2.6. SDS–PAGE and zymogram analysis for xylanolytic activities

SDS–PAGE was performed using a 12% (w/v) polyacrylamide gel with a 5% stacking gel and the Mini-Protean II system (BioRad) according to the method previously described with some modifications [27]. The sample preparation for zymogram analysis was performed by mixing 0.5 U of enzyme with 5 μl of loading buffer containing SDS 2% (w/v). The mixture was applied in a SDS-PAGE containing 1% of birchwood xylan and, after running, the gel was divided into two parts. One part, containing the molecular marker obtained from BioRad (BioRad Precision Plus Protein Unstained Standard), was stained with Comassie Brilliant Blue. The other part of the gel containing just enzymatic samples was washed twice for 30 min in 20% isopropanol (v/v) to remove SDS and allow refolding of the proteins in the gel. The gel was washed again for 30 min in 100 mM acetate buffer, pH 5, to remove 2-propanol and immediately incubated at same buffer for 15 min at 50 °C for development of xylanase activity. After that, the gel was submerged in 0.1% (w/v) Congo red solution for 30 min and destained with 1 M NaCl until pale-red hydrolysis zones appeared against a red background. Acetic acid at 0.5% concentration was added to expose the bands and the gel turned to a dark blue color.

2.7. Protein digestion and identification by mass spectrometry (MS)

Protein spots were removed manually from the gels, reduced by DTT (Dithiothreitol) and alkylated by iodoacetamide. The digestion was carried out with trypsin in a 50 mM ammonium bicarbonate buffer, pH 7.8, containing 20 ng/μl of sequencing grade trypsin (Promega) at 37 °C overnight. Peptides were extracted from the spots with 30 μl of 50 mM ammonium bicarbonate solution, followed by incubation for 10 min with occasional vortex mixing. After that, the supernatant was collected and transferred to a 0.5 ml plastic microcentrifuge tube. This extraction was performed two more times. The volume of the extract was completely dried by evaporation in a speed-vac. Tryptic peptides were solubilized in 30 μl of MS grade water (Sigma-Aldrich) containing trifluoroacetic acid 0.1% (v/v).

For protein identification by mass spectrometry, the tryptic peptides were analyzed using a MALDI-TOF/TOF mass spectrometer model Ultraflex III (Bruker Daltonics). The samples of the tryptic peptides were mixed with α-cyano-4-hydroxyl cinnamic acid in a proportion of 1:1. The mass spectra obtained were processed using FlexAnalysis software (Bruker Daltonics) and a peak list (mgf format) was used for identification of the proteins by *MS ions search* using the Mascot software against the NCBI protein databases. For the search, the following parameters were considered: a mass tolerance of 75 ppm for the parental ion, fixed modification for carbamidomethylation of cysteine residues and variable modification

for oxidation of the methionine residues. The sequences obtained were confirmed by *de novo* sequencing manually and the trypic cleavage partners of the proteins were analyzed by interpretation of the spectra using the flexAnalysis software.

2.8. Enzymatic characterization

2.8.1. Effects of pH and temperature

The influence of pH and temperature on xylanase activities was determined using the standard enzymatic assay, except that the pH values were modified to a range of 2.0–14.0, using different buffer solutions, and the temperature ranged from 25 to 70 °C.

The pH stability of xylanases was determined by pre-incubating enzyme solutions in the pH range of 2.0–14.0 for 1 h, on ice. After pre-incubation, the mixture was used for determining residual activity, according to standard assay, using xylan from beechwood as the substrate. Thermal stability was investigated by pre-incubating the enzymatic solutions in 100 mM sodium acetate buffer, pH 5.0, at temperatures of 50 and 60 °C, for periods as indicated in the text. Aliquots of the enzymes were collected at specific times and submitted to the standard assay, measuring the remaining activity. The relative activities were calculated in relation to xylanase activity without pre-incubation, which was considered to be 100%. Results of the analyses are presented as mean ± SD for three measurements.

2.8.2. Substrate specificity

Enzymatic assays were performed with various synthetic, natural and polymeric substrates. FPase and endoglucanase activities were determined using Whatman No. 1 filter paper (1 × 6 cm, 50 mg) and 1% CMC/1% Avicel® as substrates, respectively, according to previously described standard conditions [28]. The total reducing sugars liberated during the enzymatic assays were quantified by the dinitrosalicylic acid (DNS) method [26] using glucose as the standard. Xylanase activity using xylan from beechwood, oat spelt xylan and wheat arabinoxylan, all at 1% (w/v) concentration, was determined as the standard assay. β-Glucosidase, β-xylosidase, α- and β-mannosidase, α- and β-galactosidase, α-arabinofuranosidase and β-cellobiase activities were measured using ρPNβGlc, ρNPβXyl, ρNPα/βMan, ρNPα/βGal, ρNPαAra and ρNPβCel as substrates, respectively. The reaction mixtures contained 100 μl of the appropriately diluted enzyme solution, 125 μl of the synthetic substrate solution (1 mM at final concentration) and 275 μl of 100 mM sodium acetate buffer pH 5.0. The reaction mixture was incubated for 30 min and it was stopped by addition of 0.5 ml of a 0.5 M sodium carbonate solution. Absorbance was measured at 410 nm and the amount of ρ-nitrophenol released was estimated using a standard curve. The data presented for all enzyme activity determinations are mean values ± SD of three measurements.

2.8.3. Effect of ions and reducing agents

The effects of ions and reducing agents on xylanase activities were assayed by the standard method. Reaction mixtures contained 100 μl of the appropriately diluted enzyme solution with an adequate amount of the ion or reducing agent for a final concentration of 2 and 10 mM (except for β-mercapthoethanol that presented a final concentration of 1 mM) and 400 μl of xylanase solution prepared in buffer. The reaction was incubated for 15 min at 50 °C and the reducing sugars were measured at 540 nm, as described before. The data presented for all enzyme activity assays are mean values ± SD of measurements performed in triplicate.

2.8.4. Products effect

The xylooligosaccharides effect on xylanase activities (XlnB and XlnC) was evaluated through the addition of these compounds to the reaction mix described on Section 2.4. Xylotriose was used in the concentrations of 0.1 and 1 mg/ml and xylotetraose was used at 0.1 mg/ml.

2.8.5. Kinetic characterization

The kinetic parameters K_m and V_{max} of the xylanases XlnB and XlnC were estimated using Sigma Plot® 10.0. The enzymatic activity assays were performed as described on Section 2.4 with increasing concentrations of the substrates beechwood xylan, wheat arabinoxylan and oat spelt xylan. The catalytic constant (k_{cat}) was calculated dividing V_{max} by the enzyme concentration in the assay.

2.8.6. Compositional analysis of beechwood xylan hydrolysis products

The composition of hydrolysis products from 1% beechwood xylan by the xylanases XlnB and XlnC and the commercial cocktails Accellerase® 1500 and Multifect CL® was analyzed on silica gel plate (Sigma) by Thin Layer Chromatography (TLC). The reaction mixtures contained 150 μl of purified enzymes or the commercial cocktails and 300 μl of 1% beechwood xylan in 0.1 M sodium acetate buffer pH 5.0. Buffer was used for the negative control. The reaction was incubated by 20 h at 50 °C and, after this period, 25 μl of the mix was spotted on silica plate. The glass bowl (15 × 15 cm) was saturated with the mixture propanol:acetic acid:water in the ratio 1:1:0.1 (v/v). After elution, the hydrolysis products were visualized with the application of the developing solution (1% α-naphthol and 10% phosphoric acid in ethanol) and heating of the plate at 120 °C during 10 min. Xylose, xylotriose and xylotetraose solutions at 5 mg/ml concentration were used as standards.

2.9. Additive effects of the xylanase activities

To investigate the presence of additive effect between the xylanases XlnB and XlnC and the xylanase activities present in the commercial cocktails (Multifect® CL and Accellerase® 1500), xylanase assays were performed for the following mixtures: 10 FPU Multifect® CL + 15 U XlnB; 10 FPU Multifect® CL + 15 U XlnC; 10 FPU Multifect® CL + 7.5 U XlnB + 7.5 U Xun3613; 10 FPU Accellerase® 1500 + 15 U XlnB; 10 FPU Accellerase® 1500 + 15 U XlnC and 10 FPU Accellerase® 1500 + 7.5 U XlnB + 7.5 U XlnC and the measured values were obtained. The theoretical activities were calculated by the sum of the measured activities in the assays containing only the commercial cocktail and only the xylanase (individual or mixture forms). The theoretical values were compared to the measured activities and the additive effect was expressed as a percentage of the theoretical activity.

2.10. Sugarcane bagasse pretreatment

Sugarcane bagasse was washed and dried in an oven at 70 °C until reaching a constant mass, after which it was further milled (particle size less than 1 mm) and submitted to alkaline pretreatment prior to being employed in saccharification experiments. Sodium hydroxide at 1.0% (w/v) concentration was used to pretreat the milled sugarcane bagasse samples at a solid loading of 10% (w/v). The pretreatment was performed in an autoclave at 120 °C for 60 min. Pretreated bagasse was separated into solid and liquid fractions using a Buchner funnel fitted with filter paper. The solid fraction was washed thoroughly with distilled water, sealed in a hermetic vessel to retain moisture and stored at −20 °C.

2.11. Sugarcane bagasse saccharification

The purified xylanases and the commercial cocktails containing cellulases (Multifect® CL and Accellerase® 1500) were applied in a biomass saccharification experiment. Enzymatic saccharification

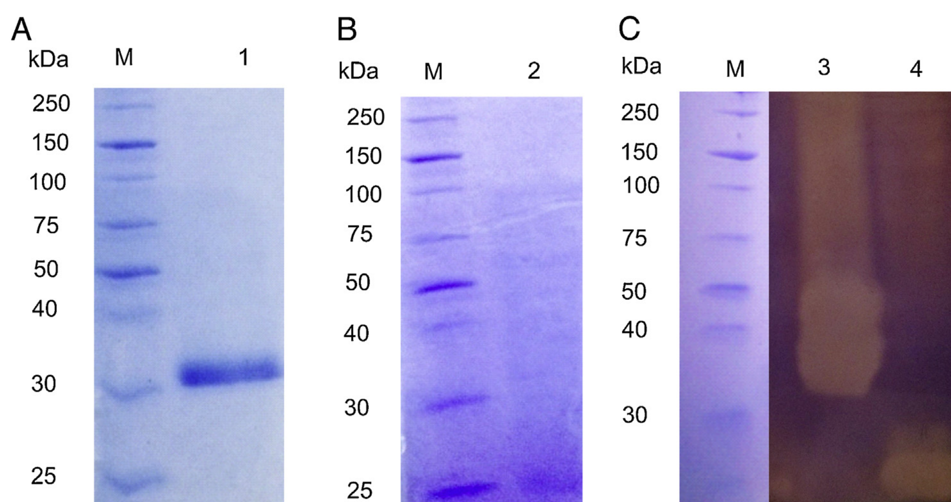

Fig. 1. (A) and (B) SDS-PAGE for xylanases extracts after affinity chromatography. (C) Zymogram of xylanases (SDS-PAGE 12% containing 1% of birchwood xylan). M – molecular mass marker stained with Comassie Brilliant Blue. 1 – Purified extract containing XlnB. 2 – Purified extract containing XlnC. 3 – Crude extract containing XlnB. 4 – Crude extract containing XlnC.

of alkali-pretreated sugarcane bagasse was performed in 125 ml Erlenmeyer flasks with 20 ml working volume, at an initial solid concentration of 8% dry matter (w/v) in 100 mM sodium acetate buffer at pH 5.0. The saccharification assays contained: the individual commercial cocktails (10 FPase units (FPU)/g of biomass); or the individual commercial cocktails (10 FPU/g biomass) supplemented with XlnB or XlnC (15 U/g biomass); or the individual commercial cocktails (10 FPU/g biomass) supplemented with XlnB and XlnC (7.5 U/g biomass for each enzyme). Sodium azide (10 mM) and tetracycline (40 μg/ml) were added to the reaction mixture to inhibit microbial contamination. The reactions were carried out in duplicate in an orbital shaker at 250 rpm and 50 °C for 72 h. Samples (0.5 ml) were taken from the reaction mixture at different time intervals for process monitoring. These samples were immediately heated to 100 °C to denature the enzymes, cooled and then centrifuged at 15,000*g* for 5 min.

2.12. Analysis of hydrolysis products

Products of the saccharification assays were analyzed by high performance liquid chromatography (HPLC) using a Shimadzu series 10A chromatograph. The HPLC was equipped with an Aminex HPX–87 P column (300 × 7.8 mm) and refractive index detector. The column was eluted with water at a flow rate of 0.6 ml/min and it operated at 80 °C.

The total reducing sugars were measured by the DNS method [26] using a mix of glucose and xylose as standard.

3. Results and discussion

3.1. Purification and biochemical characterization of XlnB and XlnC

After 72 h cultivation of *P. pastoris*, xylanase activities in the crude extract were 80.45 U/ml and 35.64 U/ml for XlnB and XlnC, respectively. Expressed in terms of the protein content, the specific activities were 311.82 U/mg of protein and 105.13 U/mg of protein for XlnB and XlnC, respectively. After purification, the xylanases exhibited a single band in SDS-PAGE, with molecular mass values of 34 kDa for XlnB (Fig. 1A) and 24 kDa for XlnC (Fig. 1B). These values are in agreement with the molecular mass expected for these proteins. The zymography showed that XlnB and XlnC were active purified xylanases (Fig. 1C).The identity of XlnB and XlnC were confirmed by mass spectrometry analysis (Fig. 2). De novo sequencing of ion *m*/*z* 2222.01 (Fig. 2B) present in MS1 of XlnB purified extract (Fig. 2A) revealed the sequence IMHWDVVNEI/LFNEDGTFR that correspond to a peptide product of tryptic hydrolysis of XlnB. Also, de novo sequencing of ion *m*/*z* 2222.01 (Fig. 2D) present in MS1 of XlnC purified extract (Fig. 2C) revealed the sequences RVSWFQEFTATGEI/L and YNAPSI/LEGTA that correspond to peptides product of tryptic hydrolysis of XlnC.

XlnB exhibited substantial activity against beechwood xylan within a pH range of 5.0-9.0 and temperature range of 50–60 °C, while XlnC showed the highest activities in the pH range 5.0-8.0 and the same temperature range. The optimal pH and temperature were 7.5 and 60 °C for XlnB, while XlnC achieved maximal activity at pH 6.0 and 50 °C (Fig. 3A–D). Since XlnB and XlnC both retained more than 75% of their activities at pH 5.0, the standard activity assay was performed at this pH value. These data are in accordance with previous studies which reported that recombinant xylanases from *Aspergillus terreus*, Plectosphaerella cucumerina, and Alternaria sp. HB186, also expressed in *P. pastoris*, showed maximal activity at pH 5.0 and 60 °C [29], pH 6.0 and 40 °C [30] and pH 6.0 and 50 °C, respectively [31]. In previous studies, *A. nidulans* xylanases XlnB (X_{34}) and (XlnC) (X_{24}), purified from *A. nidulans* had optimal pH and temperature values of 6.0/56 °C and 5.5/52 °C, respectively [20,21]. Comparison of optima pH and temperature values for the native and heterologous xylanases, showed that expression in *P. pastoris* resulted in a significant increase in the pH values at which these enzymes exhibited maximum activity, especially for XlnB which had its optimum pH changed from 6 to 7.5.

XlnB and XlnC showed significant stability over a wide pH range (Fig. 3A–B). After pre-incubation for 30 min in pH values below 4.0 and above 10.0, XlnB recovered over 65% and 90% of its initial activity, respectively, when this enzyme was returned to pH 5.0. The results show that XlnB was able to refold and retrieve its activity after pre-incubation in extreme pH ranges. Similar results were observed for XlnC that recovered over 90% of its initial activity after 30 min pre-incubation in pH values lower than 4.0 and higher than 9.0.

Concerning thermal stability (Fig. 3E–F), XlnB was very stable at 50 °C and retained about 70% of its original activity after 2 h of pre-incubation at this temperature and this residual activity was maintained until 6 h of pre-incubation. This enzyme still retained, at 50 °C, more than 30% of its original activity after 100 h of pre-incubation and, after 170 h, the residual activity was 25%. However,

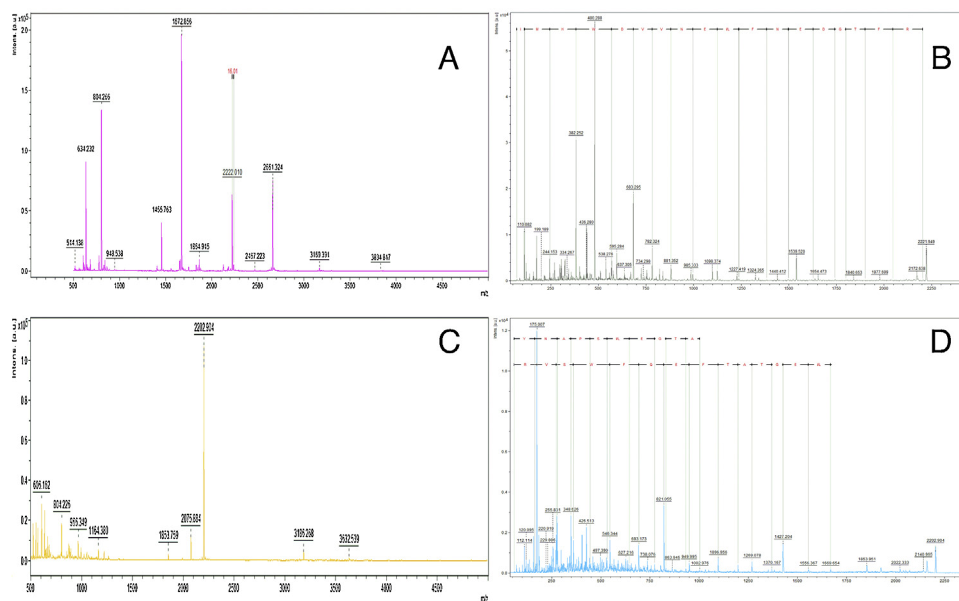

Fig. 2. Protein identification by mass spectrometry (MS). MS1 spectra of tryptic peptides of XlnB (A) and XlnC (C), respectively. De novo sequencing of the ions with *m*/*z* 2222.01 (B) and 2202.91 (D), which were obtained in MS1 of tryptic peptides of XlnB and XlnC, respectively.

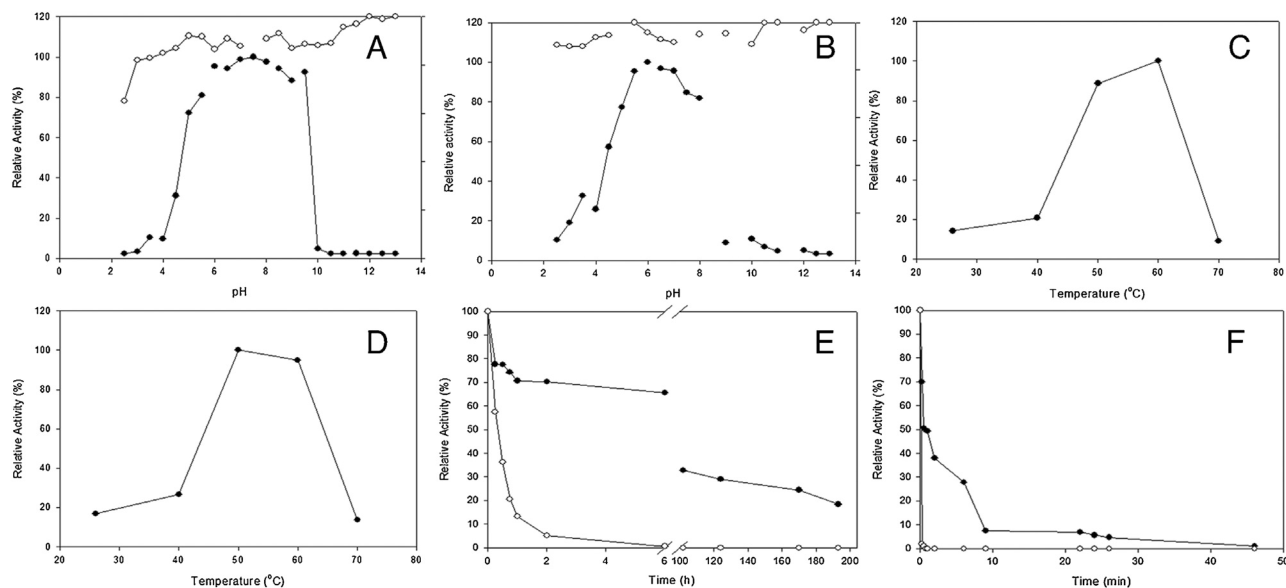

Fig. 3. Effect of pH and temperature on the xylanases from *A. nidulans*. (**A**) Effect of pH on activity (▭) and stability (●) of XlnB. (**B**) Effect of pH on activity (▭) and stability (●) of XlnC. (**C**) Effect of temperature on XlnB. Relative activities were calculated in relation to activities determined at optima pH and temperature. (**D**) Effect of temperature on XlnC. Relative activities were calculated in relation to activities determined at optima pH and temperature. (**E**) Thermostability at 50 (●) and 60 °C (▭) for XlnB. (**F**) Thermostability at 50 (●) and 60 °C (▭) for XlnC.

at 60 °C, XlnB lost activity after 9 h of pre-incubation. XlnC showed lower thermal stability compared to XlnB, since this enzyme maintained only 28% of its initial activity after 6 h of incubation at 50 °C and the activity was zero after 30 min at 60 °C. XlnB and XlnC presented half-life values of 49 h 20 min and 1 h 10 min, respectively, at 50 °C. The half-life values of XlnB and XlnC at 60 °C were drastically reduced to 20 min and 5.8 min, respectively. The higher thermostability at 50 °C and 60 °C exhibited by XlnB, which belongs to GH10, compared to XlnC, a GH11 xylanase, could be explained by the structural differences between GH10 and GH11 xylanases. GH10 Xylanases tend to show higher thermal stability since the $\alpha/\beta 8$ structure of GH10 xylanases is composed by propellers and sheets and (α/β) is the repetitive unit [32]. For the GH11 xylanases, the jelly-roll structure is composed of many sheets and only one propeller and one sheet is the repetitive unit. The propeller structures fold better than the sheet structures, which could provide higher stability of the GH10 enzymes.

The analysis of XlnB and XlnC specificities against several substrates showed that these enzymes did not hydrolyze the aryl synthetic substrates containing xylose or different monosaccharides (Suppl. Data, Table 1). On the other hand, they were able to hydrolyze different xylans. This result confirms that these enzymes exhibit endoxylanase activity, as expected, since the cloned genes correspond to GH10 and GH11 xylanases. In relation to the natural substrates, XlnB showed high activity against wheat arabinoxylan, which is a complex structure of xylan, containing many arabinofuranose decorations [33]. In contrast, XlnC had the best activity against xylan from beechwood, which is a less branched structure of

Table 1
Relative activity of XlnB and XlnC in the presence of different ions and reducing agents. Relative activities were calculated in relation to the xylanase activity without pre-incubation which was considered to be 100%.

		XlnB	XlnC
Ion/Reducing Agent	**Concentration (mM)**	**Relative Activity (%) ± DP**	
$HgCl_2$	10	0	0
	2	0	0
$ZnCl_2$	10	56.12 ± 0.01	73.65 ± 0.02
	2	58.07 ± 0.00	98.24 ± 0.02
NaCl	10	97.88 ± 0.00	93.24 ± 0.02
	2	92.76 ± 0.00	114.44 ± 0.04
$MgCl_2$	10	86.79 ± 0.03	129.97 ± 0.02
	2	83.65 ± 0.03	113.59 ± 0.01
EDTA	10	57.62 ± 0.01	76.68 ± 0.03
	2	64.34 ± 0.01	90.17 ± 0.01
SDS	10	0.00	0.00
	2	0.00	0.00
$MnCl_2$	10	93.18 ± 0.10	119.70 ± 0.02
	2	101.58 ± 0.04	116.52 ± 0.02
$CaCl_2$	10	90.28 ± 0.06	95.44 ± 0.01
	2	90.14 ± 0.03	113.21 ± 0.04
$CuSO_4$	10	0	0
	2	0	93.66 ± 0.01
β-mercapthoethanol	1	87.64 ± 0.01	122.66 ± 0.01

xylan, containing 95% of xylose [13]. Similar results were described for the natively produced enzymessince in that study XlnB did not show activity against CMC, ρNPβXil, ρNPβGli, ρNPαAra, ρNPβGal and ρNPαMan and XlnC presented higher activity against birchwood xylan, followed by arabinoxylan and insoluble oat spelt xylan [20,21].

The enzymes showed distinct sensitivities to ions and reducing agents (Table 1). XlnB and XlnC were completely inhibited by SDS and $HgCl_2$. $CuSO_4$ completely inhibited XlnB but total inactivation of XlnC was achieved only at 10 mM concentration. The denaturing action of SDS probably affected the integrity of the enzyme tridimensional structure which is fundamental for its catalytic activity [34]. The ion Cu^{2+} is known to be a strong inhibitor of xylanases [29,30]. XlnC was activated by $MnCl_2$ and $MgCl_2$, but $MgCl_2$ reduced the XlnB activity. β-Mercaptoethanol, a reducing agent, promoted the activation of XlnC which can be explained by the fact that some reduced chemical ligations in the enzyme structure are favorable for the catalytic activity [34]. The activities of native xylanases were differently affected by the presence of ions [20]. The native XlnB was completely inhibited by 1 mM of Hg^{2+} and Cu^{2+}, while the same concentration of these ions reduced the activity of native XlnC. The activity of these native enzymes were reduced by Mn^{2+} [20].

When endoxylanases act on xylan, the products are xylooligosaccharides that can inhibit the enzyme activity by a retro-inhibition mechanism [7]. The effects of the products xylotriose and xylotetraose on the activities of XlnB and XlnC showed that xylotriose did not inibit these xylanases at 0.1 mg/ml, but at 1 mg/ml XlnB activity was fully inhibited and XlnC activity was reduced to 66.7% of its original activity. Xylotetraose at 0.1 mg/ml concentration did not affect the activity of XlnB and only slightly reduced the XlnC activity to 93.3% of its original activity. This result indicates that XlnB is more sensitive to xylotriose, while XlnC was affected by xylotetraose.

The calculated values of K_m, k_{cat} and k_{cat}/K_m for the xylanases XlnB and XlnC against the different xylans are shown in Table 2. XlnB and XlnC had similar catalytic efficiency values (k_{cat}/K_m) against the tested substrates, except that XlnC was more efficient to catalyze the hydrolysis of oat spelt xylan, while XlnB hydrolyzed wheat arabinoxylan less efficiently. The kinetic constant values of native XlnB and XlnC against the different xylans [21], were compared to the values obtained for the heterologous enzymes. The native XlnB showed K_m values against birchwood xylan, arabinoxylan and oat spelts xylan of 1.78, 1.5 and 4.15 mg ml^{-1}, respectively, while these values for XlnC were 4.37, 12.14 and 12.43 mg ml^{-1}, respectively. These results suggest that the heterologous enzymes display similar kinetic properties as the native xylanases.

The hydrolysis products of beechwood xylan by XlnB and XlnC were analyzed in TLC and compared with the products obtained after beechwood xylan hydrolysis using the commercial enzymatic mixtures Accellerase® 1500 and Multifect CL® (Suppl. Data, Fig. 1A). The main product of xylan hydrolysis by XlnB and XlnC was xylotetraose, while xylotriose appeared in less concentration. The occurrence of the xylooligossacharides xylotriose and xylotetraose and the absence of xylose as the final product confirms the activity endo xylanolytic of XlnB and XlnC. The products obtained after hydrolysis by Accellerase® 1500 and Multifect CL® were xylotriose, xylotetraose and oligossacharides with a polymerization degree higher than four. The absence of standards with higher polymerization degree than xylotetraose and decorated standards prevented the identification of other xylan hydrolysis products by the tested commercial cocktails.

3.2. *Effects of XlnB and XlnC in commercial enzymatic mixtures*

XlnB and XlnC were added to the commercial enzymatic mixtures Multifect CL® and Accellerase® 1500 and the final xylanase activities were higher than the theoretical activities (Suppl. Data, Fig. 1B). The commercial cocktails already have several enzymes that are able to hydrolyze xylan, such as endoxylanases, α-arabinofuranosidases, β-xylosidases, and others [35]. Still, XlnB and XlnC had positive effect on the action of the xylanases present in the commercial cocktails. However, this effect varied depending on the commercial mixture and the use of XlnB or XlnC, probably due the mechanisms of action of XlnB (GH10) and XlnC (GH11) and the different xylanases present in Multifect CL® and Accellerase® 1500. The highest additive effect was observed for Multifect CL® supplemented with XlnB or with both enzymes (XlnB + XlnC), which were 86.53% and 86.09%, respectively. However, XlnC was more efficient for Accellerase® 1500 supplementation. These additive effects observed in beechwood xylan hydrolysis indicate that the addition of XlnB and XlnC in the commercial enzymatic mixtures used for the hydrolysis of sugarcane bagasse could improve the overall xylanolytic activity.

Table 2
Michaelis Menten constant (K_m), maximal velocity (V_{max}), catalytic constant (k_{cat}) and catalytic efficiency (k_{cat}/K_m) for XlnB and XlnC, using different xylans.

Enzyme	Substrate	Km (mg ml^{-1})	Vmax (μmol/min ml^{-1})	kcat (min^{-1})	kcat/Km (ml mg^{-1} min^{-1})
XlnB	Beechwood xylan	1.66	193.27	5.56 10^6	3.53 10^6
	Wheat arabinoxylan	6.67	626.55	1.90 10^7	2.85 10^6
	Oat spelt xylan	7.45	151.50	4.59 10^6	6.16 10^5
XlnC	Beechwood xylan	4.23	435.82	1.69 10^7	3,99 10^6
	Wheat arabinoxylan	10.27	934.03	1.10^8	1.07 10^7
	Oat spelt xylan	16.95	759.31	5.89 10^7	3.47 10^6

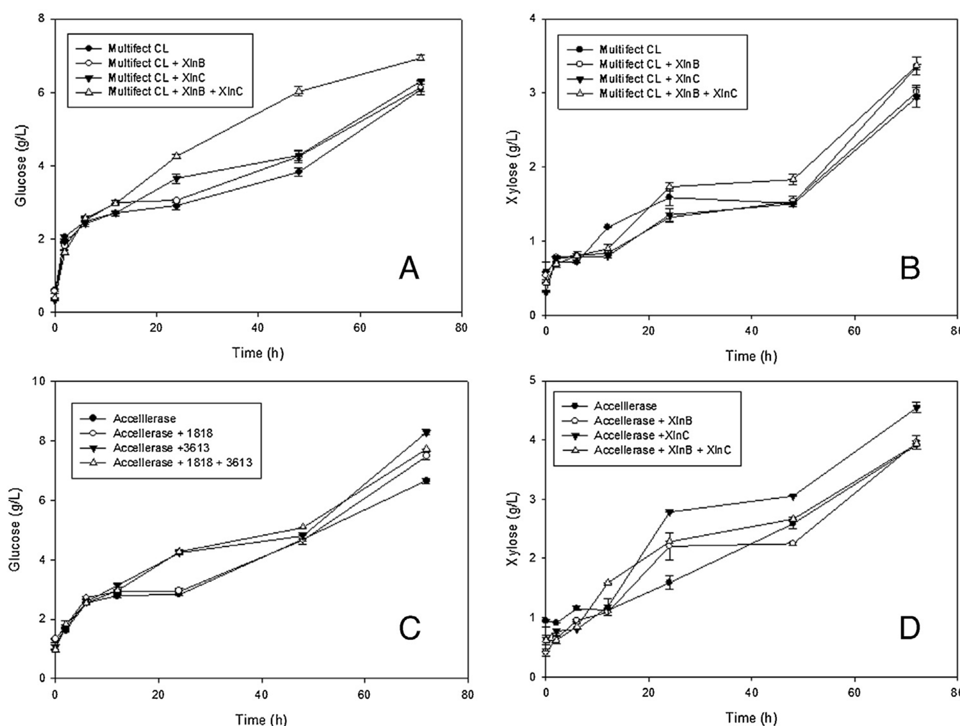

Fig. 4. Saccharification assays of alkali-pretreated sugarcane bagasse. (A) Glucose and (B) xylose release using Multifect® CL as the cellulase source. (C) Glucose and (D) xylose release using Accellerase® 1500 as the cellulase source.

3.3. Saccharification experiments

Alkali-pretreated sugarcane bagasse was hydrolyzed by commercial cocktails (Multifect® CL and Accellerase® 1500) with or without supplementation by XlnB and/or XlnC (Fig. 4).

Concerning sugarcane bagasse saccharification by Multifect® CL, a higher release of glucose (6.94 g/l) was achieved when the commercial cocktail was supplemented with both XlnB and XlnC. Xylanases are not able to produce glucose directly but these enzymes could stimulate cellulose hydrolysis since they act on the hemicellulose fraction and facilitate the access of cellulolytic enzymes to cellulose [36,37]. Multifect® CL without supplementation and with both XlnB and XlnC resulted in similar xylose release (3.36 and 3.37 g/l, respectively). Therefore the xylanases did not affect the release of xylose from oligosaccharides by the β-xylosidase present in the commercial cocktails, but likely mainly affected the xylan depolymerization.

When sugarcane bagasse saccharification was performed using Accellerase® 1500, the supplemented mixtures also resulted in higher glucose release. Accellerase® 1500 without supplementation released 6.66 g/l of this sugar, while Accellerase® 1500 with XlnC released 8.3 g/l of glucose. Supplementation with XlnC also promoted the best release of xylose (4.54 g/l). These results show that XlnC (GH11) has a better potential for sugarcane bagasse hydrolysis when used as a supplement for the Accellerase® 1500. Indeed, GH11 xylanases have for a long time been used as biotechnological tools in various industrial applications due to their interesting properties such as high substrate selectivity and high catalytic efficiency, small size (around 20 kDa) and variety of pH and temperature optimum [11]. The saccharification assays performed using Accellerase® 1500 resulted in higher release of glucose and xylose than those performed using Multifect® CL. This indicates that Accellerase® 1500 leads to higher sugar release from alkali-pretreated sugarcane bagasse, with or without supplementation.

Table 3
Total reducing sugars (the results are mean values ± SD of three measurements) and percentage of glucan and xylan conversions after 72 h of sugarcane bagasse saccharification by the different enzyme mixtures.

Enzymatic Mixture	Reducing Sugars ± SD	Saccharification Yield – Conversion	
	(μmol/ml)	Glucan (%)	Xylan (%)
Multifect CL	68.90 ± 0.03	14.03	14.45
Multifect CL + XlnB	68.36 ± 0.05	14.38	12.94
Multifect CL + XlnC	71.84 ± 0.08	14.73	12.73
Multifect CL + XlnB + XlnC	72.30 ± 0.12	16.25	14.45
Accellerase	74.93 ± 0.06	15.55	16.82
Accellerase + XlnB	79.19 ± 0.05	17.53	17.25
Accellerase + XlnC	80.33 ± 0.03	19.40	19.63
Accellerase + XlnB + XlnC	69.61 ± 0.01	18.00	17.04

Total reducing sugars were measured after 72 h of saccharification experiments (Table 3). These results confirmed that when Multifect® CL was used for sugarcane bagasse hydrolysis, the best yield was achieved by supplementation with both XlnB and XlnC and that for Accellerase® 1500 the maximal release of sugars occurred when it was supplemented with XlnC.

Complementing crude enzyme extracts shows significant promise since it can result in synergistic effects that improve the efficiency of biomass saccharification [38]. The saccharification yields in our experiments were lower than 20% which can be associated with the high solid concentration used for the experiments and also to the mild condition of alkali pretreatment. Although high solid concentration is known to decrease conversion rates, it can reduce the amount of water in the process and generate a more concentrated product, which can result in a decrease in process costs [39]. Moreover, the mild conditions are important to reduce inhibitor formation and environmental impacts but they can be associated with lower hydrolysis efficiency [40].

4. Conclusion

Two xylanases from *A. nidulans* expressed in *P. pastoris,* XlnB (GH10) and XlnC (GH11), were studied and their ability to degrade different xylans was demonstrated. The enzymes were stable in a wide pH range and reasonable temperatures and were able to act synergistically with enzymes present in commercial cellulase cocktails. They also demonstrated satisfactory results as supplements of commercial enzyme cocktails for saccharification of alkali-pretreated sugarcane bagasse. Therefore, XlnB and XlnC have a great potential for biotechnological conversions, as supplements of commercial cocktails for biomass saccharification in second generation ethanol production processes.

Conflict of interest

The authors declare no financial or commercial conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijbiomac.2016.05.065.

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