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A glucuronoxylan-specific xylanase from a new *Paenibacillus favisporus* strain isolated from tropical soil of Brazil

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Summary. A new xylanolytic strain, *Paenibacillus favisporus* CC02-N2, was isolated from sugarcane plantation fields in Brazil. The strain had a xylan-degrading system with multiple enzymes, one of which, xylanase Xyn30A, was identified and characterized. The enzyme is a single-domain xylanase belonging to family 30 of the glycosyl hydrolases (GH30). Xyn30A shows high activity on glucuronoxylans, with a Vmax of 267.2 U mg^{-1}, a K_m of 4.0 mg/ml, and a k_{cat} of 13,333 min^{-1} on beechwood xylan, but it does not hydrolyze arabinoxylans. The three-dimensional structure of Xyn30A consists of a common $(\beta/\alpha)_8$ barrel linked to a side-chain-associated β-structure, similar to previously characterized GH30 xylanases. The hydrolysis products from glucuronoxylan were methylglucuronic-acid-substituted xylooligomers (acidic xylooligosaccharides). The enzyme bound to insoluble xylan but not to crystalline cellulose. Our results suggest a specific role for Xyn30A in xylan biodegradation in natural habitats. The enzyme is a good candidate for the production of tailored xylooligosaccharides for use in the food industry and in the biotechnological transformation of biomass. [**Int Microbiol** 2014; 17(3):175-184]

Keywords: *Paenibacillus favisporus* · xylanase · glycosyl hydrolases GH30

Introduction

Xylan is an abundant component of the plant cell wall and the major component of hardwood hemicelluloses. The biodegradation of xylan is a complex process that requires the coordinated activity of several enzymes, including xylanases (EC 3.2.1.8), which play a key role by catalyzing the hydrolysis of internal linkages in the β-1,4-xylose backbone of the polymer. Xylanases are produced by fungi, bacteria, and protozoa [10,31] and are currently used in a wide range of industrial applications, such as the food and textile industries, in wastewater management, and in pulp bleaching, all of which exploit the robust activity of xylanases under extreme conditions, including those of industrial processes [10,33]. Xylanases are currently the subject of intense research into the bioconversion of lignocellulosic biomass for use as biofuels and high-added-value products [22,46,47].

Xylanases are grouped into different families according to their amino acid sequence, structural fold, and catalytic mechanism [9]. Most of the xylanases characterized to date belong to glycoside hydrolase (GH) families GH10 and GH11. They hydrolyze different types of xylan, including arabinoxylans, glucuronoxylans, and even algal β-1,3-β-1,4-xylan [3,31]. A few xylanases specific for glucuronoxylans have been characterized. These belong to GH family 30 (GH30) and their

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activity requires methylglucuronic acid (MeGlcA) side chains [36,42,45]; for this reason they are referred to as glucuronoxylanases. A common feature of their 3D structure is a typical $(\beta/\alpha)_8$ barrel catalytic domain [34] fused to a side β-structure of nine strands that seems to be required for catalytic activity [42]. Xylanases also occur in the GH8 family [11,13] and a xylanase of family GH5 specific for arabinose-substituted xylan was recently characterized [12]. The cooperation and synergism between xylanases from different families facilitate the degradation of plant xylan in natural habitats and the complete depolymerization of lignocellulosic biomass.

With their great biodiversity, the soils of the Brazilian Atlantic tropical region are a potential source of as yet uncharacterized microorganisms, including lignocellulolytic bacteria belonging to the phyla Proteobacteria, Actinobacteria, and Firmicutes [8]. The diversity of microorganisms present in soils is a notable source of industrial enzymes [20]. With the aim of identifying new xylanases with biotechnological potential, we screened Brazilian tropical soils for xylan-depolymerizing microorganisms and thereby isolated a new bacterial strain, tentatively classified as *Paenibacillus favisporus*. The bacterium has a multiple xylanase system resembling those described in members of the genus *Paenibacillus* [5,21]. In this study, we cloned, purified, and characterized a new xylanase from *P. favisporus*.

Materials and methods

Isolation of the microbial strain. Soil (25 g) from a sugarcane plantation field in Brazil (6.59 S 35.1 W) was used as a screening source. The soil was suspended in 225 ml of Ringer solution containing (g/l): NaCl, 2.250; KCl, 0.105; $CaCl_2$, 0.120; $NaHCO_3$, 0.050. The suspension was mixed by rotation on an orbital shaker (Certomat RM, B. Braun Biotech Intl.) at 200 rpm for 20 min. Suitably diluted samples were spread onto solid medium containing (g/l): beechwood xylan (Sigma-Aldrich, St. Louis, MO), 1.0; $NaNO_3$, 0.5; K_2HPO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.5; $FeSO_4 \cdot 7H_2O$, 0.01; yeast extract, 1.0; and agar, 15.0; at pH 7.0. The plates were incubated at 55 °C for 3 days. Isolates were transferred into xylan-containing agar medium and incubated at 55 °C for 3 days. Microorganisms with xylanolytic activity were detected based on the formation of clear zones around colonies, visualized using the Congo red staining method [38]. Enzyme production was studied in Erlenmeyer flask cultures (100 ml) containing 20 ml of LB medium and (g/l): yeast extract, 5.0; NaCl, 10; tryptone, 10; rice straw, 10.0. The flasks were inoculated with 0.5% of the overnight inoculum and incubated at 37ºC under shaking (200 rpm) for 48 h. Cultures were centrifuged at 10,000 ×*g* at 4ºC for 20 min, and the cell-free clear supernatant was analyzed in a zymogram assay. Isolate CC02-N2 was chosen for subsequent studies.

Nucleic acid manipulation. Bacterial genomic DNA was extracted with the High Pure PCR product purification kit (Roche) according to the manufacturer's instructions. Genomic DNA was diluted appropriately and used as a template in polymerase chain reactions (PCRs) with the universal bacterial primers 27F and 1525R. The PCRs were run for 25 cycles with the following thermal profile: 94ºC for 1 min, 55ºC for 1 min, and 72ºC for 2 min and final extension for 10 min at 72 °C. The PCR amplicon was sequenced using an ABI BigDye Terminator v3.1 cycle sequencing kit and an ABI 3730XL DNA analyzer (Applied Biosystems, USA).

Degenerate primers deduced from the sequence of the gene encoding xylanase Xyn30D from *Paenibacillus barcinonensis* [42] allowed amplification of a portion of a xylanase gene from isolate CC02-N2. The complete sequence of the gene, *xyn30A*, was obtained by gene walking from the DNA amplified fragment using the Genome Walker universal kit (Clontech).

To overexpress the xylanase, *xyn30A* was PCR amplified (Kapa-HiFi, KAPA Biosystems) with the oligonucleotide primers FW 5′-AACTATGATTC TATCAAAGAGAATGGAG-3′) and BW (5′-GTGGTGGTGATGGTGATGG CCATGCGCCAATTCACCTACGAA-3′) and cloned into pLATE31 (Thermo Scientific), giving rise to recombinant plasmid pLATE31-Xyn30A, which produced the full length enzyme, containing the signal peptide, linked to a C-terminal His6 tag (Xyn30A). All DNA constructs were verified by sequencing. Sequence homology was analyzed by BLAST [3].

Expression and purification of recombinant proteins in *E. coli*. Xyn30A was purified from *E. coli* BL21 Star (DE3) recombinant clones containing plasmid pLATE31-Xyn30A. Exponential-phase cultures (OD_{600} 0.6) were induced with 1 mM isopropyl-β-D-thiogalactopyranoside at 37°C for 3 h. The cells were disrupted in a French press. The recombinant His6-tagged protein was purified from cell extracts by immobilized metal affinity chromatography using 1-ml HisTrapHP columns (GE Healthcare) and elution in 20 mM phosphate buffer (pH 7.0) with a 0–500 mM imidazole gradient. An additional purification step was performed by gel filtration in Superdex 200/10-300 columns of 24 ml (GE Healthcare) on a fast protein liquid chromatography system (ÄKTA FPLC; GE Healthcare). Buffer exchange and protein concentration were performed in Centricon centrifugal filter units of 10-kDa molecular mass cutoff (Millipore).

Enzyme assays. Xylanase activity was assayed by measuring the amount of reducing sugar released from xylan from hardwoods or cereals by the Nelson-Somogyi method [33]. The standard assay was performed at 50°C in phosphate buffer (pH 6.5) for 15 min as described previously [42]. Birchwood, beechwood, and oat spelt xylans and 4-*O*-methyl-glucuronoxylan were purchased from Sigma-Aldrich. Rye and wheat arabinoxylans were purchased from Megazyme. One unit of enzymatic activity was defined as the amount of enzyme that releases 1 µmol of reducing sugar equivalent per min under the assay conditions described. A standard curve of xylose was used to calculate activity units. The protein concentrations of the samples were determined using the Bradford method [6]. All determinations of enzyme activity were done in triplicate.

The effects of temperature and pH on xylanase activity were evaluated by response surface methodology (RSM) using 22 central composite designs with 5 coded levels leading to 11 sets of experiments, 8 unique combinations, and 3 replications at the central point . The Briton-Robinson buffer, in a pH range between 4.0 and 11.0 [7], and temperatures ranging from 50 to 90ºC were used in the analysis.

The influence of metal ions and chemicals on xylanase activity was determined by incorporating $AlCl_3$, $BaCl_2$, $CaCl_2$, $CuSO_4$, $FeSO_4$, $HgCl_2$, KCl, $LiCl_2$, $MgCl_2$, $MnCl_2$, NaCl, NH_4Cl_2, or $ZnSO_4$ at a concentration of 10 mM; and EDTA, SDS, Tween 80, Triton X-100 or DMSO at a concentration of 0.5% into the reaction mixture. Relative xylanase activity was determined.

The kinetics of Xyn30A were characterized in terms of Michaelis-Menten kinetic constants (K_m and Vmax) by assaying the enzyme activity on beechwood xylan at concentrations of 1.3–40.0 mg/ml under conditions of maximum activity. Enzyme kinetics were analyzed using GraphPad software version 4.0.

Gel electrophoresis and zymograms. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 12% gels, essentially as described [23]. For the detection of xylanase activity, 0.2% birchwood xylan was included in the gels before polymerization, and zymograms were developed as described [41]. The samples were heated at 50ºC for 15 min in sample buffer before their application to the gels. After electrophoresis, the gels were washed in 2.5% triton X-100 for 30 min, incubated in 200 mM phosphate buffer at pH 6.5 for 30 min, and finally incubated at 55ºC for 10 min in the same pre-warmed fresh buffer. In some cases, phosphate buffer at pH 6.5 was replaced by 200 mM acetate buffer pH 5.0, 200 mM Tris-HCl buffer pH 7.0, or 200 mM Tris-glycine buffer pH 9.0. The gels were stained with 0.1% Congo red for 15 min, washed with 1M NaCl until the xylanase bands became visible, immersed in 10% (v/v) acetic acid, and then photographed.

Binding to insoluble polysaccharides. Binding activity to insoluble polysaccharides was assayed as described by Hogg et al. with some modifications [18]. Briefly, 250 µg of purified Xyn30A was mixed with 25 mg of Avicel or insoluble oat spelt xylan in a final volume of 500 µl of 50 mM phosphate buffer (pH 6.5) in 1.5-ml microcentrifuge tubes. The samples were incubated at 4°C for 1 h with gentle orbital mixing and then centrifuged at 18,000 ×*g* for 20 min. The supernatants, containing unbound protein, were carefully removed. The pellets were washed three times with 400 µl of the same buffer, resuspended in 400 µl of 10% SDS, and heated at 100ºC for 10 min to release bound protein. The samples were then analyzed by SDS-PAGE on 10–15% polyacrylamide gels.

Analysis of the hydrolysis products from xylan and xylo-oligosaccharides. Thin-layer chromatography (TLC) was performed as previously described [15]. Xyn30A (1.7 µM) was incubated with 1.5% birchwood or beechwood xylan at 65°C in 50 mM phosphate buffer (pH 6.0) for 18 h. The reactions were stopped by heating at 90°C for 15 min. All samples and markers were adjusted to pH 6.0 before loading. For the analysis of xylan hydrolysis products by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS), 1 µl of the hydrolysates was mixed with 1 µl of matrix solution [10 mg/ml 2,5- dihydroxybenzoic acid dissolved in acetonitrile-water (1:1, vol/vol), 0.1% (wt/vol) trifluoroacetic acid]. One µl of the mixture was spotted onto the MALDI-TOF MS plate and allowed to dry before the analysis. Positive mass spectra were collected with a 4800 Plus MALDI TOF/TOF (ABSciex 2010) spectrometer with an Nd:YAG 200-Hz laser operated at 355 nm.

Sequence analysis. The 16S rRNA gene sequences of related taxa were obtained from GenBank. Phylogenetic trees were constructed using the neighbor-joining method with the program MEGA 5.0 [37]. BLAST searches were performed for DNA or protein sequence analysis, including domain classification. A putative signal peptide was identified through PrediSi [http://www.predisi.de] [17]. The 3D structure homology models were generated with SwissModel software [http://swissmodel.expasy.org] based on template *gtnA*, corresponding to XynC from *Bacillus subtilis* 168. Pymol software (PyMOL Molecular Graphics System, version 1.2r3pre; Schrödinger) was used to visualize the 3D protein models. The ExPASy proteomics server [http://us.expasy.org/tools/protparam.html] was used to analyze the protein physicochemical parameters (ProtParam tool).

Nucleotide sequence accession number. The DNA sequences of the 16S rRNA gene and *xyn30A* gene of *P. favisporus* CC02-N2 were submitted to the GenBank database under accession numbers KF442953 and KF442954, respectively.

Results and Discussion

Isolation and characterization of *Paenibacillus favisporus* CC02-N2. Strain CC02-N2 was isolated from the soil of a sugarcane plantation in Northeastern Brazil and selected for its high hydrolytic activity on xylan. Culture of the strain on rice-straw-supplemented LB for 48 h resulted in the secretion of 6.95 xylanase U/ml, whereas endoglucanase activity on carboxymethyl cellulose was not detectable. The composition of the xylan-degrading system of the strain was studied by zymographic analysis of the culture supernatants. Several xylanase bands, ranging from 20 to 135 kDa, all of them showing activity in the pH range 5.0–9.0, were detected in the gels (Fig. 1). Based on the multiple xylanase system of the strain CC02-N2 and the high production of xylanase activity, devoid of significant activity on cellulose, it was chosen for further study.

The analysis of its 16S rDNA sequence revealed that strain CC02-N2 belonged to the genus *Paenibacillus*, with high phylogenetic relatedness to *Paenibacillus favisporus* GMP01 (99.4% 16S rDNA gene identity) [43] and *Paenibacillus cineris* LMG18439 (99.3% identity) [25], isolated from cow

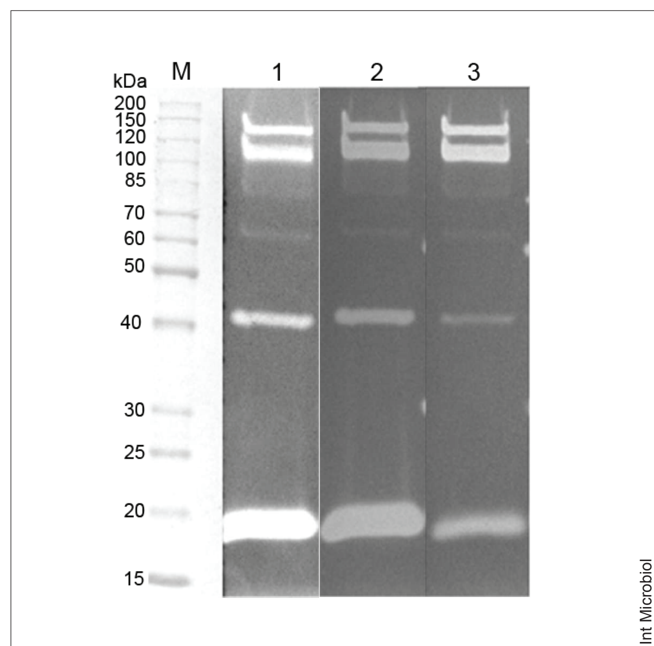

Fig. 1. Zymogram analysis of xylanases from strain CC02-N2. Crude supernatants of strain CC02-N2 cultures in 1% rice-straw-supplemented LB. The zymograms were performed at: (1) pH 5.0; (2) pH 7.0; and (3) pH 9.0. (M) position of the mass standards.

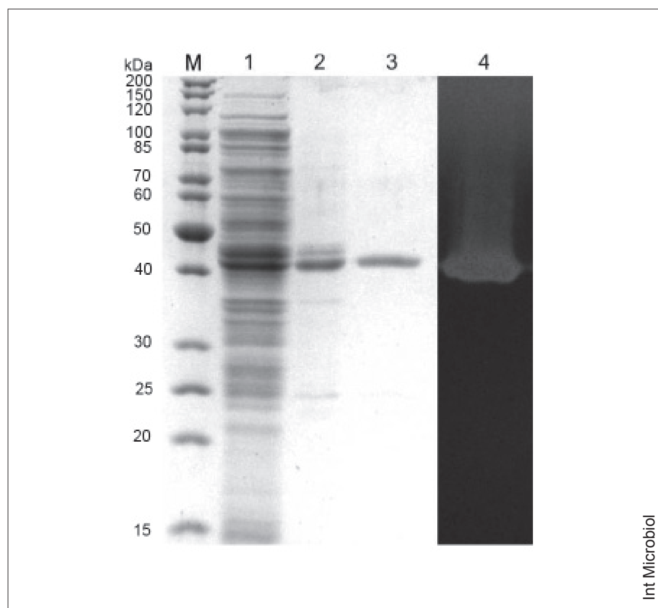

Fig. 2. SDS-PAGE analysis of purified Xyn30A. (1) Cell extracts, (2) active fraction from His-Trap chromatography, (3) purified Xyn30A from Superdex 200/10-300 gel filtration columns, (4) zymogram of xylanase activity, (M) position of the mass standards.

feces and Antarctic volcanic soils, respectively. A phylogenetic tree illustrating the relationship of strain CC02-N2 to closely related species was constructed (data not shown). In accordance with its 16S rRNA gene identity to known species, the isolated strain was tentatively classified as *Paenibacillus favisporus* CC02-N2, although additional analysis will be required for its full taxonomic characterization.

Cloning and sequence analysis of Xyn30A. The above-described results suggested that *Paenibacillus favisporus* strain CC02-N2 secreted xylanases from different GH families, as reported for *Paenibacillus barcinonensis* [5,15, 42]. Among these enzymes, a glucuronoxylanase from family GH30 was recently characterized [42]. In light of the novelty of xylanases specific for MeGlcA branched xylan and their synergism with GH11 xylanases in pulp bleaching [14], we searched for these enzymes in *P. favisporus* CC02-N2. Using degenerate primers deduced from *P. barcinonensis* Xyn30D, a fragment of the coding region of a xylanase from *P. favisporus* CC02-N2 was amplified. By gene walking of this DNA fragment, the sequence of a 1,432-bp DNA fragment containing a 1,290-bp open reading frame (ORF) encoding a xylanase of 430 amino acids was determined. This ORF has an N-terminal region of 29 amino acids with the features of a signal peptide. The predicted molecular mass and isoelectric point of the deduced mature protein were 47,972.9 Da and 6.8, respectively. Sequence analysis by comparison to proteins contained in the NCBI database showed that the cloned enzyme was a single-domain xylanase belonging to GH30. The cloned enzyme, named Xyn30A, showed high homology to hypothetical xylanases from *Paenibacillus terrae* HPL-003 [32] and *Paenibacillus polymyxa* E681 [21] (84% and 82% identity, respectively) and to the GH30 domains of the well characterized Xyn30D from *P. barcinonensis* and XynC from *B. subtilis* (81% and 80% identity, respectively), whereas homology to *Erwinia chrysanthemi* XynA was lower (39% identity).

Characterization of Xyn30A. Xyn30A was cloned into plasmid (pLATE31) under the control of the T7 promoter in *E. coli* BL21 Star (DE3) to produce a fusion protein of full-length Xyn30A and a C-terminal His tag. The recombinant protein Xyn30A was purified to homogeneity from cell extracts by two-step chromatography using HisTrapHP and Superdex 200/10-300 columns. The purified Xyn30A was analyzed by SDS-PAGE and zymography, which showed that it had an apparent molecular mass of 47.9 kDa, in accordance with that deduced from the amino acid sequence (Fig. 2).

The substrate specificity of Xyn30A was determined by evaluating the activity of the enzyme on xylans and other polysaccharides. The enzyme showed high hydrolytic activity on glucuronoxylans. Beechwood xylan was the preferred substrate, yielding a specific activity of 244 xylanase U/mg (Table 1). By contrast, the enzyme did not show detectable activity on arabinoxylans from oat spelt, rye, and wheat, nor did it show activity on any of the other polysaccharides tested, including crystalline or amorphous celluloses. The substrate specificity of Xyn30A was similar to that of the four GH30 glucuronoxylanases characterized to date: *E. chrysanthemi*

Table 1. Substrate specificity of Xyn30A

Substrate	Activity (U/mg)
Beechwood xylan	244.0 ± 4.1
Birchwood xylan	202.5 ± 11.4
4-*O*-methyl-glucuronoxylan	225.1 ± 18.3
Oat spelt xylan	ND[a]
Wheat arabinoxylan	ND
Rye arabinoxylan	ND
Carboxymethyl cellulose	ND
Avicel	ND

[a]ND, not detected.

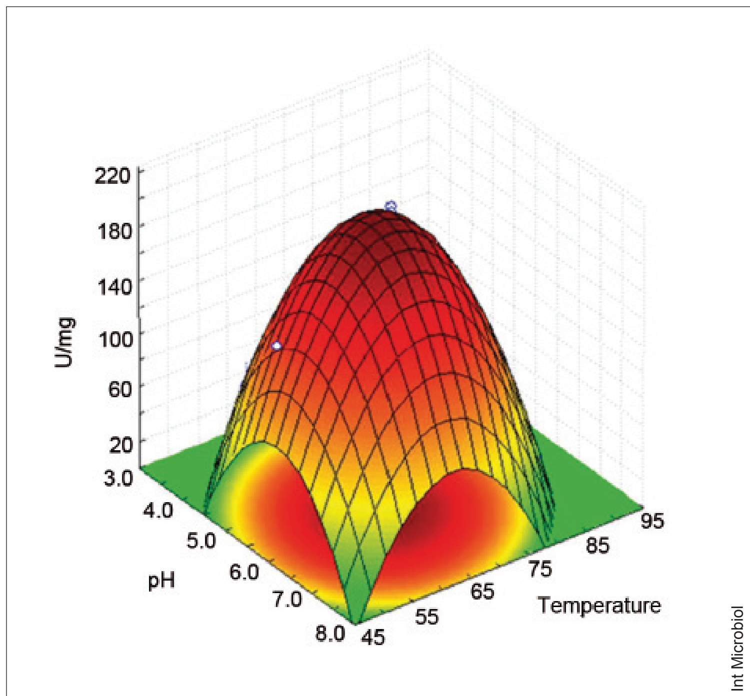

Fig. 3. Response surface plot showing the effect of temperature and pH on the xylanase activity of Xyn30A. The dots represent the averages of three replicates.

XynA [19,45], *B. subtilis* XynC [36], Xyn30B from *Bacillus* sp. BP-7 [14], and *P. barcinonensis* Xyn30D [42]. All of these enzymes are specific for glucuronoxylans, as a result of their requirement for MeGlcA branches for catalysis, and are not active on arabinoxylans.

The effect of temperature and pH on xylanase activity and the interaction between the two factors were determined by RSM (Fig. 3). The conditions that led to maximum activity were 65ºC and pH 6.0. Thermostability assays showed that Xyn30A remained highly stable up to 50ºC after 3 h of incubation at pH 6.0; about 60% of the original activity was maintained after incubation under these conditions but at at 60ºC (Fig. 4).

An analysis of the kinetic variables of Xyn30A on beech-

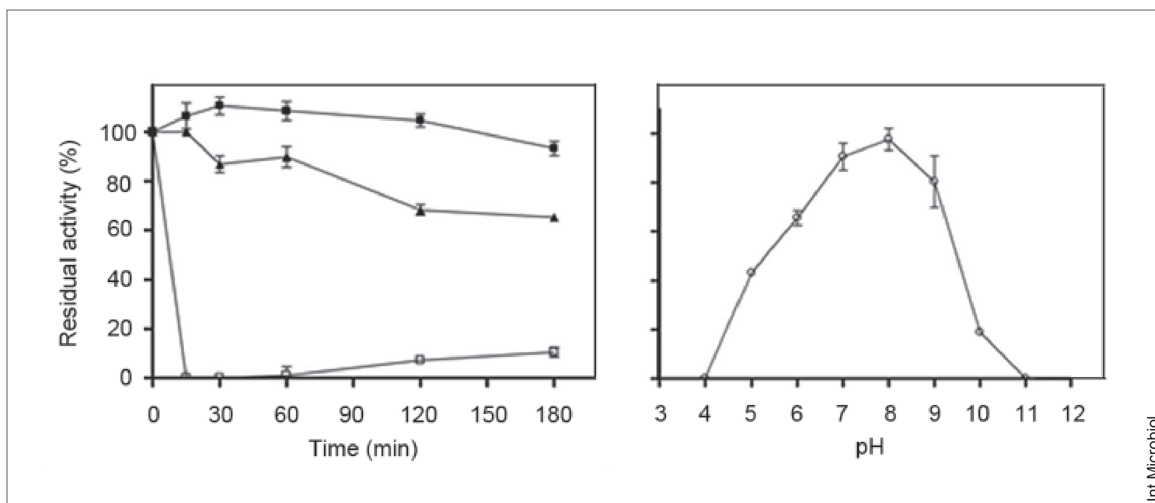

Fig. 4. Effect of temperature and pH on the stability of Xyn30A. (**A**) The samples were incubated in 50 mM phosphate buffer (pH 6.0) at 50ºC (■), 60ºC (▲) and 70ºC (□), and residual activity after different time intervals was determined. (**B**) The samples (○) were incubated at 65ºC in buffers at different pH for 3 h, after which residual activity was determined.

Table 2. Effect of metal ions and other compounds on the activity of Xyn30A

	Relative activity (%)
Control	100 ± 1.4
Al^{3+}	0.0
Ba^{2+}	93.2 ± 8.9
Ca^{2+}	86.4 ± 13.4
Cu^{2+}	5.8 ± 0.8
Fe^{2+}	6.9 ± 0.3
Hg^{2+}	0.6 ± 0.2
K^{+}	106.0 ± 1.2
Li^{+}	100.9 ± 5.3
Mg^{2+}	103.3 ± 0.5
Mn^{2+}	38.5 ±0.6
Na^{+}	96.5 ± 0.8
NH_4^{+}	101.4 ± 3.4
Zn^{2+}	7.7 ± 0.5
EDTA	99.1 ± 4.0
SDS	6.1 ± 3.1
Tween 80	26.6 ± 8.2
Triton X-100	11.5 ± 0.0
DMSO	92.4 ± 1.0

wood xylan showed a Vmax of 267.2 U mg^{-1} and a K_m of 4.0 mg/ml at optimal conditions for activity. Comparison of the kinetic constants of Xyn30A with those reported for GH30 xylanases showed that the K_m of Xyn30A was higher than that of XynC from *B. subtilis* (1.63 mg/ml) but lower than that of Xyn30D from *P. barcinonensis* (14.72 mg/ml). A larger difference was found among the k_{cat} values of these enzymes. Thus, while *B. subtilis* XynC and *P. barcinonensis* Xyn30D had k_{cat} values of 2,635 and 1,510 min^{-1}, respectively, the k_{cat} of Xyn30A was 13,333 min^{-1}, much higher than the values of the GH30 xylanases characterized so far.

The effect of different metal ions and chemicals on Xyn30A activity was also determined (Table 2). The enzyme was completely inhibited by Al^{3+} and strongly inhibited by Hg^{2+}, Cu^{2+}, Fe^{2+}, Zn^{2+}, Mn^{2+}, Triton X-100, Tween 80, and SDS. Inhibition by Ba^{2+}, Ca^{2+}, Na^{+}, and DMSO was minimal. Li^{+}, NH^{4+}, and EDTA had no effect on enzyme activity, whereas K^{+} and Mg^{2+} produced a small stimulating effect. The inhibitory effect of Al^{3+} on Xyn30A resembles that reported for several amylases, in which aluminum ions were shown to inactivate the enzymes, presumably by binding to catalytic residues [2,29].

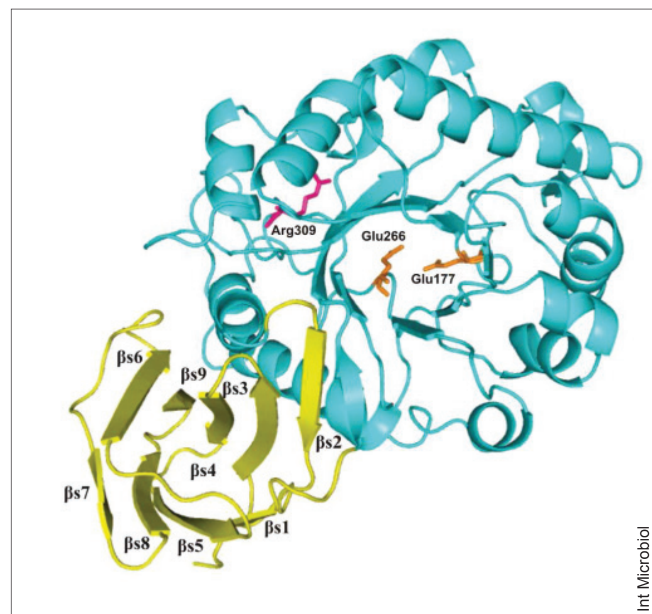

Fig. 5. Computer modeling of the 3D structure of Xyn30A. The (β/α)8 barrel is shown in cyan and the associated β-domain in yellow. The numbering of the βs strands and amino acids is indicated.

All GH30 glucuronoxylanases share a unique overall 3D structure of the catalytic module, which consists of a (β/α)8 barrel fused to a side β-structure of nine strands, referred to as the side-associated β-domain [24,34]. Computer modeling of Xyn30A based on the crystalline structure of *B. subtilis* XynC showed a similar 3D structure of a (β/α)8 barrel with an associated β-domain (Fig. 5). Valenzuela et al [42] reported that deletion of this side structure abolishes the activity of *P. barcinonensis* Xyn30D, evidence of the importance of the β-side structure for the catalytic activity of GH30 xylanases. A comparison to sequences of *Bacillus subtilis* XynC and *Erwinia chrysanthemi* XynA identified the glutamic acid residues (Glu177 and Glu266) in the catalytic groove of Xyn30A. These residues are thought to provide the catalytic acid/base and nucleophile of GH30 glucuronoxylanases (Fig. 5). The two enzymes also contain a conserved arginine, Arg303 in *B. subtilis* XynC and Arg293 in *E. chrysanthemi* XynA, responsible for MeGlcA side-chain recognition in glucuronoxylan, which determines the substrate specificity of these xylanases [35,40]. The conserved arginine was also found in the corresponding location (Arg309) of Xyn30A. Two recently characterized GH30 xylanases from fungal microorganisms differ from the aforementioned enzymes in their activity on both

glucoronoxylan and arabinoxylan [27,39] Alignment of the respective amino acid sequences showed that these fungal enzymes do not have the conserved arginine residue proposed to recognize glucuronoxylan [39]. In a dendrogram analysis of GH30 xylanases, the fungal xylanases clustered separately from glucuronoxylanases. In fact, according to St John et al. [34], the fungal enzymes can be placed in a different subgroup of GH30 xylanases, acidic xylanases, which are active on glucuronoxylan and arabinoxylan and show an acidic pH optimum for activity [27,39].

Products of xylan degradation by Xyn30A. The hydrolysis products from glucuronoxylans were analyzed by TLC. Beechwood xylan was degraded to a mixture of products of intermediate mobility between linear xylooligosaccharides, indicating that they were MeGlcA-substituted xylooligomers, while xylose and linear oligosaccharides were not found among the hydrolysis products (Fig. 6). To better characterize the products of beechwood xylan hydrolysis, they were also analyzed by MALDI-TOF MS. The mass spectra showed the presence of molecular ions of substituted xylooligomers and their sodium salts, identified as sodium adducts (Table 3). The major ions corresponded to substituted xylooligosaccharides consisting of 3–8 xylopyranosyl residues and a single MeGlcA residue, in accordance with the results of the TLC analysis. The results also agree with the mode of action of GH30 xylanases, which cleave the bonds at the second position after the MeGlcA branches, giving rise to xyloligosaccharides with a substitution at the penultimate xylose residue from the reducing end [36,45].

Table 3. Oligosaccharides identified by MALDI-TOF MS after the hydrolysis of beechwood xylan by Xyn30A

Xylooligomer	M_r[a]	m/z Na
$MeGlcAX_2$	472	–
$MeGlcAX_3$	604	627.2
$MeGlcAX_4$	736	759.2
$MeGlcAX_5$	868	891.3
$MeGlcAX_6$	1000	1023.3
$MeGlcAX_7$	1132	1155.4
$MeGlcAX_8$	1264	1287.4

[a]M_r, molecular weight.

Binding to polysaccharides. Binding of Xyn30A to Avicel and insoluble oat spelt xylan was studied as described in Materials and methods. As expected, Xyn30A did not bind to Avicel. Most of the enzyme remained in the supernatants and only a very small amount was detected adsorbed on Avicel. By contrast, the enzyme bound to insoluble oat spelt xylan, as most of the enzyme was found adsorbed on the polymer while only a small amount remained in the supernatants (Fig. 7). Xyn30A is a single-domain enzyme devoid of a carbohydrate binding module (CBM). However, our results clearly showed that the enzyme bound to insoluble xylans, implying a polysaccharide binding ability of its catalytic GH30 domain. Reported studies of xylan binding of *P. barcinonensis* Xyn30D, a modular GH30 xylanase that includes a CBM35, have shown that not only the whole enzyme and its CBM but also the isolated GH30 domain binds to insoluble xylan [42]. Polysaccharide binding of single-domain xylanases was previously reported for *Bacillus circulans* BcX, a GH11 xylanase thought to contain a secondary xylan-binding site that overcomes the lack of a CBM [26]. The observed binding ability of the GH30 catalytic module could be attributed to substrate binding site of the catalytic groove, or to the β-side associated domain, which as mentioned above seems to be required for the catalytic activity of GH30 xylanases. In

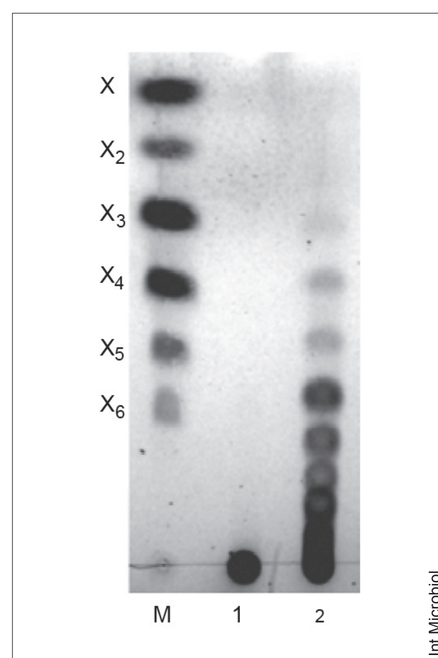

Fig. 6. Thin-layer chromatography analysis of thte hydrolysis products from beechwood xylan. (1) Control, no digested samples; (2) products of xylan hydrolysis by Xyn30A; (M) size markers of xylose (X), xylobiose (X_2), xylotriose (X_3), xylotetraose (X_4), xylopentaose (X_6), and xylohexaose (X_6).

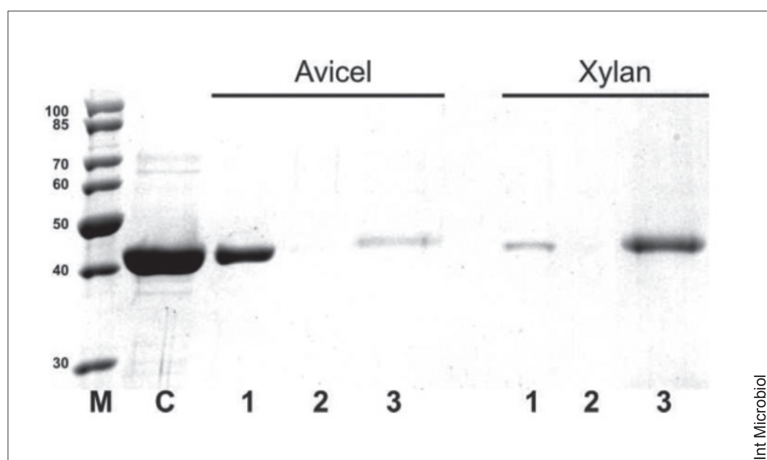

Fig. 7. SDS-PAGE analysis of the binding of Xyn30A to insoluble polysaccharides. Proteins were mixed with Avicel or with the insoluble fraction of oat spelt xylan for 1 h; bound and unbound fractions were separated by centrifugation and analyzed by SDS-PAGE. (1) Unbound fraction, (2) wash, (3) fraction absorbed to the polymer, (C) control protein, (M) position of the mass standards.

fact crystallographic analysis of XynC from *Bacillus subtilis* identified ligand coordination to its β-side domain, which has been proposed as a new type of CBM [35].

The enzyme characterized in this study is one of the few glucuronoxylanases of the GH30 family described so far. The enzyme's lack of activity on arabinoxylans has probably hindered the identification of these enzymes, which may complement the activity of GH10 and GH11 xylanases on lignocellulosic biomass. The recent identification of several GH30 glucuronoxylanases, including *P. favisporus* Xyn30A, suggests that these enzymes are widespread among gram-positive xylanolytic bacteria [14,42]. Their unique mode of action on glucuronoxylans suggests their use to selectively modify xylan and in the design of tailored oligomers. These MeGlcA-substituted xylooligosaccharides (acidic XOS) have also been proposed as emerging prebiotics, as they were shown to selectively stimulate the growth of probiotic microorganisms, including *Bifidobacterium,* which ferment them to short-chain fatty acids [1,30]. Hardwood xylan can provide a source of acidic XOS as food additives and nutraceuticals [16,28]. In those reports, xylan was depolymerized by chemical means, and the resulting XOS was refined to eliminate subproducts such as xylose and other monosaccharides. Enzymatic treatment as an alternative for XOS production could diminish subproduct formation. In addition, GH30 glucuronoxylanases are unique because xylose is not released from xylan, thus avoiding further processing of the hydrolysates. The high k_{cat} of Xyn30A makes this enzyme of particular interest among GH30 xylanases for the production of acidic XOS and for the transformation of biomass. Glucuronoxylanases can be important tools in biorefinery approaches to obtain added-value products from abundant raw materials, such as *Eucalyptus* wood, whose potential based on its high xylan content is usually underestimated. Further research is required to ascertain the role of GH30 glucuronoxylanases on xylan hydrolysis. Analysis of the function of their distinctive β-side domain in substrate recognition and catalysis will provide insights into its contribution to xylan degradation in natural habitats.

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References

1. Aachary AA, Prapulla SG (2011) Xylooligosaccharides (XOS) as an emerging prebiotic: microbial synthesis, utilization, structural characterization, bioactive properties, and applications. Compr Rev Food Sci Food Saf 10:2-16
2. Aguilar G, Morlon-Guyot J, Trejo-Aguilar B, Guyot J (2000) Purification and characterization of an extracellular α-amylase produced by *Lactobacillus manihotivorans* LMG 18010^{T}, an amylolytic lactic acid bacterium. Enzyme Microb Technol 27:406-413
3. Altschul S (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389-3402
4. Biely P, Vršanská M, Tenkanen M, Kluepfel D (1997) Endo-β-1,4-xylanase families: differences in catalytic properties. J Biotechnol 57:151-166
5. Blanco A, Vidal T, Colom JF, Pastor FI (1995) Purification and properties of xylanase A from alkali-tolerant *Bacillus* sp. strain BP-23. Appl Environ Microbiol 61:4468-4470

6. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-254
7. Britton HTS (1952) Hydrogen ions, 2nd ed. London
8. Bruce T, Martinez IB, Maia Neto O, Vicente ACP, Kruger RH, Thompson FL (2010) Bacterial community diversity in the Brazilian Atlantic forest soils. Microb Ecol 60:840-849
9. Cantarel BL, Coutinho PM, Rancurel C, Bernard T, Lombard V, Henrissat B (2009) The Carbohydrate-Active EnZymes database (CAZy): an expert resource for glycogenomics. Nucleic Acids Res 37:233-238
10. Collins T, Gerday C, Feller G (2005) Xylanases, xylanase families and extremophilic xylanases. FEMS Microbiol Rev 29:3-23
11. Collins T, Meuwis M-A, Stals I, Claeyssens M, Feller G, Gerday C (2002) A novel family 8 xylanase, functional and physicochemical characterization. J Biol Chem 277:35133-35139
12. Correia MAS, Mazumder K, Brás JLA, Firbank SJ, Zhu Y, Lewis RJ, York WS, Fontes CMGA, Gilbert HJ (2011) Structure and function of an arabinoxylan-specific xylanase. J Biol Chem 286:22510-22520
13. Fushinobu S, Hidaka M, Honda Y, Wakagi T, Shoun H, Kitaoka M (2005) Structural basis for the specificity of the reducing end xylose-releasing exo-oligoxylanase from *Bacillus halodurans* C-125. J Biol Chem 280:17180-17186
14. Gallardo O, Fernández-Fernández M, Valls C, Valenzuela SV, Roncero MB, Vidal T, Díaz P, Pastor FIJ (2010) Characterization of a family GH5 xylanase with activity on neutral oligosaccharides and evaluation as a pulp bleaching aid. Appl Environ Microbiol 76:6290-6294
15. Gallardo O, Pastor FIJ, Polaina J, Diaz P, Łysek R, Vogel P, Isorna P, González B, Sanz-Aparicio J (2010) Structural insights into the specificity of Xyn10B from *Paenibacillus barcinonensis* and its improved stability by forced protein evolution. J Biol Chem 285:2721-2733
16. Gullón P, González-Muñoz MJ, Gool MP van, Schols HA, Hirsch J, Ebringerová A, Parajó JC (2011) Structural features and properties of soluble products derived from *Eucalyptus globulus* hemicelluloses. Food Chem 127:1798-1807
17. Hiller K, Grote A, Scheer M, Münch R, Jahn D (2004) PrediSi: prediction of signal peptides and their cleavage positions. Nucleic Acids Res 32:375-379
18. Hogg D, Pell G, Dupree P, Goubet F, Martín-Orúe SM, Armand S, Gilbert HJ (2003) The modular architecture of *Cellvibrio japonicus* mannanases in glycoside hydrolase families 5 and 26 points to differences in their role in mannan degradation. Biochem J 371:1027-1243
19. Hurlbert JC, Preston JF 3rd (2001) Functional characterization of a novel xylanase from a corn strain of *Erwinia chrysanthemi*. J Bacteriol 183:2093-2100
20. Juturu V, Wu JC Microbial xylanases: engineering, production and industrial applications. Biotechnol Adv 30:1219-1227
21. Kim JF, Jeong H, Park S-Y, Kim S-B, Park YK, Choi S-K, Ryu C-M, Hur C-G, Ghim S-Y, Oh TK, Kim JJ, Park CS, Park S-H (2010) Genome sequence of the polymyxin-producing plant-probiotic rhizobacterium *Paenibacillus polymyxa* E681. J Bacteriol 192:6103-6104
22. Kumar S, Singh SP, Mishra IM, Adhikari DK (2009) Recent advances in production of bioethanol from lignocellulosic biomass. Chem Eng Technol 32:517-526
23. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685
24. Larson SB, Day J, Barba de la Rosa AP, Keen NT, McPherson A (2003) First crystallographic structure of a xylanase from glycoside hydrolase family 5: implications for catalysis. Biochemistry 42:8411-8422
25. Logan NA, De Clerck E, Lebbe L, Verhelst A, Goris J, Forsyth G, Rodríguez-Díaz M, Heyndrickx M, De Vos P (2004) *Paenibacillus cineris* sp. nov. and *Paenibacillus cookii* sp. nov., from Antarctic volcanic soils and a gelatin-processing plant. Int J Syst Evol Microbiol 54:1071-1076
26. Ludwiczek ML, Heller M, Kantner T, McIntosh LP (2007) A secondary xylan-binding site enhances the catalytic activity of a single-domain family 11 glycoside hydrolase. J Mol Biol 373:337-354
27. Luo H, Yang J, Li J, Shi P, Huang H, Bai Y, Fan Y, Yao B (2010) Molecular cloning and characterization of the novel acidic xylanase XYLD from *Bispora* sp. MEY-1 that is homologous to family 30 glycosyl hydrolases. Appl Microbiol Biotechnol 86:1829-1839
28. Moure A, Gullón P, Domínguez H, Parajó JC (2006) Advances in the manufacture, purification and applications of xylo-oligosaccharides as food additives and nutraceuticals. Process Biochem 41:1913-1923
29. Nirmala M, Muralikrishna G (2003) Three α-amylases from malted finger millet (Ragi, *Eleusine coracana*, Indaf-15)—purification and partial characterization. Phytochemistry 62:21-30
30. Ohbuchi T, Takahashi T, Azumi N, Sakaino M (2009) Structual analysis of neutral and acidic xylooligosaccharides from hardwood kraft pulp, and their utilization by intestinal bacteria in vitro. Biosci Biotechnol Biochem 73:2070-2076
31. Pollet A, Delcour JA, Courtin CM (2010) Structural determinants of the substrate specificities of xylanases from different glycoside hydrolase families. Crit Rev Biotechnol 30:176-191
32. Shin SH, Kim S, Kim JY, Song HY, Cho SJ, Kim DR, Lee K-I, Lim HK, Park N-J, Hwang IT, Yang K-S (2012) Genome sequence of *Paenibacillus terrae* HPL-003, a xylanase-producing bacterium isolated from soil found in forest residue. J Bacteriol 194:1266
33. Spiro RG (1966) The Nelson-Somogyi copper reduction method. Analysis of sugars found in glycoproteins. In: Elizabeth F. Neufeld VG (ed) Methods in Enzymology, Academic Press, pp 3-26
34. St John FJ, González JM, Pozharski E (2010) Consolidation of glycosyl hydrolase family 30: A dual domain 4/7 hydrolase family consisting of two structurally distinct groups. FEBS Lett 584:4435-4441
35. St John FJ, Hurlbert JC, Rice JD, Preston JF, Pozharski E (2011) Ligand bound structures of a glycosyl hydrolase family 30 glucuronoxylan xylanohydrolase. J Mol Biol 407:92-109
36. St John FJ, Rice JD, Preston JF (2006) Characterization of XynC from *Bacillus subtilis* subsp. *subtilis* strain 168 and analysis of its role in depolymerization of glucuronoxylan. J Bacteriol 188:8617-8626
37. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731-2739
38. Teather RM, Wood PJ (1982) Use of Congo red-polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. Appl Environ Microbiol 43:777-780
39. Tenkanen M, Vršanská M, Siika-aho M, Wong DW, Puchart V, Penttilä M, Saloheimo M, Biely P (2013) Xylanase XYN IV from *Trichoderma reesei* showing exo- and endo-xylanase activity. FEBS J 280:285-301
40. Urbániková L, Vršanská M, Mørkeberg Krogh KBR, Hoff T, Biely P (2011) Structural basis for substrate recognition by *Erwinia chrysanthemi* GH30 glucuronoxylanase. FEBS J 278:2105-2116
41. Valenzuela S V, Díaz P, Pastor FIJ (2010) Recombinant expression of an alkali stable GH10 xylanase from *Paenibacillus barcinonensis*. J Agric Food Chem 58:4814-4818

42. Valenzuela SV, Diaz P, Pastor FIJ (2012) Modular glucuronoxylan-specific xylanase with a family CBM35 carbohydrate-binding module. Appl Environ Microbiol 78:3923-3931
43. Velázquez E, de Miguel T, Poza M, Rivas R, Rosselló-Mora R, Villa TG (2004) *Paenibacillus favisporus* sp. nov., a xylanolytic bacterium isolated from cow faeces. Int J Syst Evol Microbiol 54:59-64
44. Vieille C, Zeikus GJ (2001) Hyperthermophilic enzymes: sources, uses, and molecular mechanisms for thermostability. Microbiol Mol Biol Rev 65:1-43
45. Vršanská M, Kolenová K, Puchart V, Biely P (2007) Mode of action of glycoside hydrolase family 5 glucuronoxylan xylanohydrolase from *Erwinia chrysanthemi*. FEBS J 274:1666-1677
46. Wang J, Sun B, Cao Y, Tian Y, Wang C (2009) Enzymatic preparation of wheat bran xylooligosaccharides and their stability during pasteurization and autoclave sterilization at low pH. Carbohydr Polym 77:816-821
47. Zhao X, Cheng K, Liu D (2009) Organosolv pretreatment of lignocellulosic biomass for enzymatic hydrolysis. Appl Microbiol Biotechnol 82:815-827