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Two Family 11 Xylanases from *Achaetomium* sp. Xz-8 with High Catalytic Efficiency and Application Potentials in the Brewing Industry

Liang Zhao,[†,‡,∥] Kun Meng,[†,∥] Yingguo Bai,[†] Pengjun Shi,[†] Huoqing Huang,[†] Huiying Luo,[†] Yaru Wang,[†] Peilong Yang,[†] Wei Song,[‡] and Bin Yao*[,†]

[†]Key Laboratory for Feed Biotechnology of the Ministry of Agriculture, Feed Research Institute, Chinese Academy of Agricultural Sciences, Beijing 100081, People's Republic of China

[‡]College of Life Science, Capital Normal University, Beijing 100048, People's Republic of China

ABSTRACT: This study identified two family-11 xylanase genes (*xynC81* and *xynC83*) in *Achaetomium* sp. Xz-8, a thermophilic strain from a desert area with substantial xylanase activity, and successfully expressed them in *Pichia pastoris*. Their deduced amino acid sequences showed the highest identity of ≤90% to known fungal xylanases and of ≤62% with each other. The purified recombinant xylanases showed optimal activities at pH 5.5 and 60−65 °C and exhibited stability over pH 5.0−10.0 and temperatures at 55 °C and below. XynC81 had high catalytic efficiency (6082 mL/s/mg), and XynC83 was favorable for xylooligosaccharide production. Under simulated mashing conditions, combination of XynC83 and a commercial β-glucanase improved the filtration rate by 34.76%, which is much better than that of Novozymes Ultraflo (20.71%). XynC81 and XynC83 had a synergistic effect on viscosity reduction (7.08%), which is comparable with that of Ultraflo (8.47%). Thus, XynC81 and XynC83 represent good candidates for application in the brewing industry.

KEYWORDS: *Achaetomium, xylanase, heterologous expression, catalytic efficiency, brewing*

INTRODUCTION

Endo-1,4-β-xylanase (EC 3.2.1.8) is the crucial enzyme that randomly cleaves the β-1,4-glycosidic linkages of the xylan backbone, releasing xylooligomers of different lengths. According to the amino acid sequence-based classification of glycoside hydrolases (GH) (http://www.cazy.org/fam/acc_GH.html),[1] enzymes with xylanase activity belong to families 5, 7, 8, 10, 11, and 43. Compared to other counterparts, xylanases of family 11 have several interesting properties, such as high substrate selectivity and catalytic efficiency, relatively small size (around 20 kDa), and variety of pH and temperature optima, making them attractive and suitable for various applications.[2]

Xylan is a major structural polysaccharide of plant cell walls and accounts for approximately 33% of all renewable organic carbon on earth.[3] For efficient conversion of xylan into economically valuable products, such as xylooligosaccharide, xylulose, xylitol, and ethanol, more and more research is being done to explore novel xylanases with specific potentials for application in animal feed, breadmaking, paper and pulp industry, waste treatment, brewing, and bioethanol.[4,5]

A majority of microorganisms, including bacteria, yeasts, and fungi, produce large amounts of and many different types of xylanases. Most of these xylanases are thermoliable or of low efficiency, thus failing to meet the demands of industrialization.[2,6] Thermophilic fungi that produce varieties of high-temperature-active enzymes are excellent microbial sources of thermostable/thermotolerant xylanases.[7] The genus *Achaetomium* consists of 25 recorded species to date (http://www.indexfungorum.org/Names/Names.asp), and most of them are isolated from soil. This genus was first described by Rai et al. in 1967, and the type species was *Achaetomium globosum*.[8] Until now, only one thermophilic species, *Achaetomium thermophilum*, has been reported.[9]

In this study, we obtained a strain of the genus *Achaetomium* from a desert sand sample and cloned two xylanase genes from it. Their gene products were successfully expressed in *Pichia pastoris*, and the recombinant proteins showed superior properties, such as broad pH and temperature adaptability and stability, less complex hydrolysis products, and high catalytic efficiency. Their potential use in the brewing process was also evaluated.

MATERIALS AND METHODS

Microorganism Isolation. The sand sample was collected from a desert area in Yinchuan, China, with a systematic sampling grid (8 cm in diameter and 5 cm in depth). One gram of sample was suspended in 9 mL of sterile saline solution (0.75% NaCl) and serially diluted, and 0.1 mL of the dilution was spread onto PDA (BD/Difco, Sparks, MD, USA) plates. After growth at 45 °C for 3 days, the isolates were purified and screened on agar plates containing 0.5% (w/v) beechwood xylan at 45 °C for 5 days. The plates were flooded with 0.1% Congo red and destained with 1 M NaCl as Wood et al. described.[10] Only strains having extracellular xylanase activities showed clear zones. The strain with the most significant xylanase activity, designated Xz-8, was identified on the basis of the morphology of fungal culture as well as the sequences of ITS-5.8S rDNA, β-tubulin gene, RNA polymerase II second largest subunit gene, and translation elongation factor 1α gene.[11,12]

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Table 1. Primers Used in This Study

primers	sequences (5′→3′)[a]	size (bp)
XynC81-yzF	ATGGTCACCTTCTCGTCCCTCTTCCTCGCTG	31
XynC81-yzR	TTAGAGGCACTGCGAGTACCACTGGTTGGCG	31
XynC83-yzF	ATGGTCTCCCTCAAGGCCGTTCTTCTCGGCG	31
XynC83-yzR	TTACGAGACGGTGATAGACGAAGAACCGCTGCTCTG	36
XynC81-expF	GGGGAATTCGCCCCCGGCGAGCTTCCGGGCA	31
XynC81-expR	GGGGCGGCCGCTTAGAGGCACTGCGAGTACCACTGGTTG	42
XynC83-expF	GGGGAATTCTTCCCGTTCAACGTCACGCAGATGAACG	37
XynC83-expR	GGGGCGGCCGCTTACGAGACGGTGATAGACGAAGAACCGC	42

[a]Restriction sites incorporated into the primers are underlined.

Strains, Plasmids, Culture Media, and Chemicals. *Escherichia coli* Trans1-T1 and the plasmid pEASY-T3 for gene cloning and sequencing were purchased from TransGen (Beijing, China). *P. pastoris* GS115 and the plasmid pPIC9 from Invitrogen (Carlsbad, CA) were used for heterologous expression. *E. coli* Trans1-T1 and *P. pastoris* GS115 were cultivated in Luria−Bertani (LB) medium at 37 °C and yeast peptone dextrose (YPD) medium at 30 °C, respectively.

Birchwood xylan, beechwood xylan, barley β-glucan, carboxymethyl cellulose sodium (CMC-Na), and Avicel were purchased from Sigma-Aldrich (St. Louis, MO, USA). Soluble and insoluble wheat arabinoxylans were obtained from Megazyme (Wicklow, Ireland). The DNA purification kit and LA *Taq* DNA polymerase were purchased from TaKaRa (Dalian, China). The restriction endonucleases and T4 DNA ligase were from Thermo Fisher Scientific (Waltham, MA, USA) and Promega (Madison, WI, USA), respectively. All chemicals were of analytical grade and commercially available.

Cloning of the Xylanase Genes from Strain Xz-8. Genomic DNA of strain Xz-8 was extracted and purified with a Fungal DNA Isolation Kit (Omega Biotek, Norcross, GA, USA). The core regions of the encoding genes were amplified by degenerate primers X11-F and X11-R specific for GH 11 xylanases[13] with the genomic DNA of strain Xz-8 as the template. The PCR products were ligated into the pEASY-T3 vector for sequencing and analyzed using BLASTx (http://www.ncbi.nlm.nih.gov/BLAST/). The 5′ and 3′ flanking regions of the core regions were obtained by using the fusion primer and nested integrated PCR (FPNI-PCR)[14] and assembled with the known core regions by using the ContigExpress software (Invitrogen).

To induce xylanase production, strain Xz-8 was grown in the medium containing 5 g/L NaCl, 5 g/L $(NH_4)_2SO_4$, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4{\cdot}7H_2O$, 0.2 g/L $CaCl_2$, 0.01 g/L $FeSO_4{\cdot}7H_2O$, and 50 g/L wheat bran for 3 days at 45 °C. The mycelia were collected, and the total RNA was extracted and purified using the SV Total RNA Isolation System (Promega) according to the manufacturer's instructions. The full-length cDNAs of xylanase-encoding genes were amplified from total RNA by RT-PCR with the ReverTra Ace-α-TM kit (TOYOBO, Osaka, Japan), fast-pfu DNA polymerase (Transgen, Beijing, China) and two specific primer sets (Table 1). The specific PCR products were purified and ligated into the pEASY-T3 vector for sequencing.

Sequence Analysis. DNA and protein sequences were aligned using the BLASTn and BLASTp programs (http://www.ncbi.nlm.nih.gov/BLAST/), respectively. Genes, introns, exons, and transcription initiation sites were predicted using the online software FGENESH (http://linux1.softberry.com/berry.phtml). The signal peptide was predicted using SignalP 4.0 Server (http://www.cbs.dtu.dk/services/SignalP/). The potential N-glycosylation sites were predicted online (http://www.cbs.dtu.dk/services/NetNGlyc/). Multiple sequence alignments were performed with ClustalW.[15] Homology modeling and calculations of protein ionization and electrostatic potential were performed using Accelrys Discovery Studio software (DS 2.5, http://www.accelrys.com).

Heterologous Expression of *xynC81* and *xynC83* in *P. pastoris*. The gene fragments coding for mature XynC81 and XynC83 without the signal peptide sequences were amplified with expression primers as shown in Table 1. The PCR products were digested with *Eco*RI and *Not*I and then cloned into the pPIC9 vector in-frame fusion of the α-factor signal peptide to construct the recombinant plasmids. The recombinant plasmids, pPIC9-*xynC81* and pPIC9-*xynC83*, were linearized using *Bgl*II and transformed into *P. pastoris* GS115 competent cells, respectively, by using a Gene Pulser Xcell Electroporation System (Bio-Rad, Hercules, CA, USA). The positive transformants were screened on the basis of their enzymatic activities in shake tubes. The ones with highest activities were selected for fermentation in 1 L shake flasks following the method described by Qiu et al.[16]

Purification of Recombinant Xylanases. The 2-day-old *P. pastoris* cultures induced by 0.5% methanol were collected by centrifugation at 12000*g* for 10 min at 4 °C. The cell-free culture supernatants were then concentrated with a Vivaflow 50 ultrafiltration membrane of 5 kDa molecular weight cutoff (Vivascience, Hannova, Germany) and loaded onto a HiTrap SP Sepharose XL FPLC column (GE Healthcare, Uppsala, Sweden) for XynC81 and a Superdex 75 10/300 GL column (GE Healthcare) for XynC83. The HiTrap SP Sepharose XL FPLC column was equilibrated with buffer A (20 mM McIlvaine buffer, pH 6.0), and proteins were eluted using a linear gradient of NaCl (0−1.0 M) in the same buffer at a flow rate of 3 mL/min. The Superdex 75 10/300 GL column was equilibrated with McIlvaine buffer (50 mM, pH 6.0), and proteins were eluted using the same buffer at a flow rate of 0.5 mL/min. The fractions exhibiting xylanase activities were collected and subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) analysis.[17] The protein concentrations were determined according to the Bradford method[18] using a protein assay kit (Bio-Rad) with bovine serum albumin as the standard. The purified recombinant enzymes were deglycosylated with endo-β-*N*-acetylglucosaminidase H (Endo H) according to the manufacturer's instructions (New England Biolabs, Ipswich, MA, USA). Both the deglycosylated and untreated enzymes were analyzed by SDS-PAGE.

Xylanase Activity Assay. The xylanase activity was determined according to a 3,5-dinitrosalicylic acid (DNS) method.[19] The standard reaction system consisted of 100 μL of properly diluted enzyme and 900 μL of 100 mM McIlvaine buffer (pH 5.5) containing 1% (w/v) beechwood xylan at 50 °C for 10 min. The reaction was terminated by the addition of 1.5 mL of DNS reagent. The mixture was boiled for exactly 5 min and cooled to room temperature, and its absorbance at 540 nm was measured. The reaction system with the same enzyme sample added after DNS reagent was treated as a control. All assays were run in triplicate. One unit of xylanase activity was defined as the amount of enzyme that released 1 μmol of reducing sugar per minute from the substrate equivalent to xylose under the assay conditions (pH 5.5, 50 °C, 10 min).

Biochemical Characterization. Beechwood xylan was used as the substrate for enzyme characterization. The optimal pH of enzyme

activity was determined at 50 °C for 10 min in buffers over the pH range from 3.0 to 12.0. The buffers used were 100 mM McIlvaine buffer (pH 3.0−8.0), 100 mM Tris-HCl (pH 8.0−9.0), and 100 mM glycine−NaOH (pH 9.0−12.0). For the pH stability assay, enzymes were incubated in buffers of pH 3.0−11.0 at 37 °C for 1 h without the substrate, and then the residual enzyme activity was measured at pH 5.5 and 50 °C for 10 min.

The optimal temperature for enzyme activity was examined at pH 5.5 for 10 min over the temperature range from 30 to 90 °C. For the thermal stability, the enzymes were incubated without substrate at 55, 65, or 75 °C for 2, 5, 10, 15, 20, 30, and 60 min, and the residual enzyme activity was measured under the standard conditions.

To determine the effect of different metal ions and chemical reagents on the enzyme activities of purified recombinant XynC81 and XynC83, 1 and 5 mM concentrations of various metal ions (Na^+, K^+, Li^+, Ag^+, Ca^{2+}, Co^{2+}, Cr^{3+}, Ni^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Pb^{2+}, Hg^{2+}, and Fe^{3+}) and chemical reagents (SDS, EDTA, and β-mercaptoethanol) were added to the reaction system individually. The system without any additive was used as a control, and each assay was measured under the standard assay conditions.

Kinetic Analysis. The K_m and V_{max} values for the purified recombinant enzymes were determined from Lineweaver−Burk plots using the nonlinear regression computer program GraFit (version 7, Erithacus Software, Horley, UK). The enzyme activities were assayed at 50 °C in McIlvaine buffer (pH 5.5) for 5 min containing 0.1−1% (w/v) beechwood xylan as the substrate. Each experiment was repeated three times, and each experiment included three replicates.

Substrate Specificity and Analysis of Hydrolysis Products. The substrate specificities of the purified recombinant XynC81 and XynC83 were determined in the standard assay system containing one of the following substrates (1%; w/v): birchwood xylan, beechwood xylan, soluble and insoluble wheat arabinoxylan, barley β-glucan, CMC-Na, and Avicel.

To analyze their hydrolysis products, reaction systems as described by Li et al.[20] were used with some modifications. Extra enzyme (10 U) and 100 μg of substrate (birchwood xylan, beechwood xylan, or soluble wheat arabinoxylan) in 140 μL of McIlvaine buffer (pH 5.5) were incubated at 50 °C for 12 h. After removal of the enzyme from the reaction using the Amicon Ultra-0.5 Centrifugal Filter Unit with Ultracel-3 membrane (Millipore, Billerica, MA, USA), the hydrolysis products of each reaction were analyzed by high-performance anion-exchange chromatography (HPAEC) with a model 2500 system from Dionex (Sunnyvale, CA, USA). Xylose, xylobiose, xylotriose, and xylotetraose were used as standards.

Effects of Xylanases on the Filtration Rate and Viscosity of Mash. Preparation of the mash and assays to determine the filtration rate and viscosity followed the methods described by Bai et al.[21] Reactions contained 50 mL of mash and 1.0 mL of each xylanase (40 U), the commercial β-glucanase Glu-66 (40 U) from Xiasheng (Ningxia, China), the xylanase combination (20 U of each xylanase), the enzyme combinations of each xylanase (20 U of XynC81 or XynC83), and Glu-66 (20 U) or the commercial enzyme complex Ultraflo (40 U) from Novozymes (Copenhagen, Denmark). The reaction containing 1.0 mL of McIlvaine buffer (100 mM, pH 5.5) instead of enzyme solution was used as the control. The reduction of the filtration time and the viscosity were calculated using the following equations:[22]

$$\Delta\psi = \frac{(\psi_{control} - \psi) \times 100}{\psi_{control}}$$

ψ is the total flow time of 5 mL mash, and $\Delta\psi$ is the filtration time reduction.

$$\mu = \frac{(\mu_{water} \times t \times \rho)}{(t_{water} \times \rho_{water})}$$

$$\Delta\mu = \frac{(\mu_{control} - \mu) \times 100}{\mu_{control}}$$

μ is the viscosity, t is the total flow time through the viscometer, $\Delta\mu$ is the viscosity reduction, and ρ is the density.

Nucleotide Sequence Accession Numbers. The nucleotide sequences of the ITS-5.8S rDNA, β-tubulin gene, RNA polymerase II second largest subunit gene, translation elongation factor 1α gene, and the two GH 11 xylanase genes *xynC81* and *xynC83* of *Achaetomium* sp. Xz-8 were deposited in the GenBank database under accession numbers KC254730, KF042858, KF042859, KF042860, KC254725, and KC254726, respectively.

RESULTS

Microorganism Isolation. The harsh desert climate is generally dry and hot, and the temperature of the sand surface at the sampling site reaches up to 50 °C during the daytime. Such an environment is ideal for the isolation of thermophiles. Among all of the fungal isolates, strain Xz-8 showed the largest clear zone on beechwood xylan agar plates with Congo red dying, that is, had the most significant xylanase activity. Strain Xz-8 showed optimal growth at 45 °C and grew better at temperatures above 45 °C than at 25 °C, suggesting that strain Xz-8 is a typical thermophilic fungus.

The strain of Xz-8 was classified as a strain of *Achaetomium* by the molecular phylogeny tree using the ITS-5.8S rDNA sequence, the β-tubulin gene, the RNA polymerase II second largest subunit gene, and the translation elongation factor 1α gene of the strain Xz-8 and those of *A. globosum* (type species of the genus of *Achaetomium*), *Achaetomium crystallim*, *Achaetomium luteum*, *Achaetomium strumarium* and *Chaetomium luteum* of the Centraalbureau Voor Schimmelcultures Fungal Biodiversity Center (CBS) (data not shown). *Achaetomium* sp. Xz-8 was deposited in the China General Microbiological Culture Collection Center under registration number CGMCC 6545.

Gene Cloning and Sequence Analysis. Two different fragments were amplified from the genomic DNA of *Achaetomium* sp. Xz-8 by degenerate primers of X11-F and X11-R specific for GH 11 xylanase genes. The 5′ and 3′ flanking regions of each core region were obtained by using FPNI-PCR and were assembled with the core regions to obtain two full-length genomic sequences, 1080 bp for *xynC81* and 722 bp for *xynC83*. Three (85, 71, and 75 bp, respectively) and one (62 bp) intron interrupted the coding sequences of *xynC81* and *xynC83*, respectively. A putative signal peptide was identified at the N-terminus of each xylanase, residues 1−19 for XynC81 and residues 1−16 for XynC83. The calculated molecular masses were 27.5 kDa for XynC81 and 21.9 kDa for XynC83. Both xylanases had putative N-glycosylation sites, one (Asn 91) for XynC81 and two (Asn 20 and Asn 90) for XynC83.

Sequence analysis indicated that XynC81 consists of a catalytic domain of GH 11 at the N-terminus and a cellulose binding module (CBM) of family 1 at the C-terminus, whereas XynC83 contains only a catalytic domain of GH 11. Although both XynC81 and XynC83 were from the same microbial source and belong to the same family, they showed significant divergence with an identity of <62% to each other. The deduced sequence of XynC81 showed the highest identity of 83% with the GH 11 endo-β-1,4-xylanase (CgXB; BAA08650.1) from *Chaetomium gracile* and 69% with the GH 11 xylanase Xyn11A from *Chaetomium thermophilum* with crystal structure (PDB 1XNK).[23] Deduced XynC83 exhibited

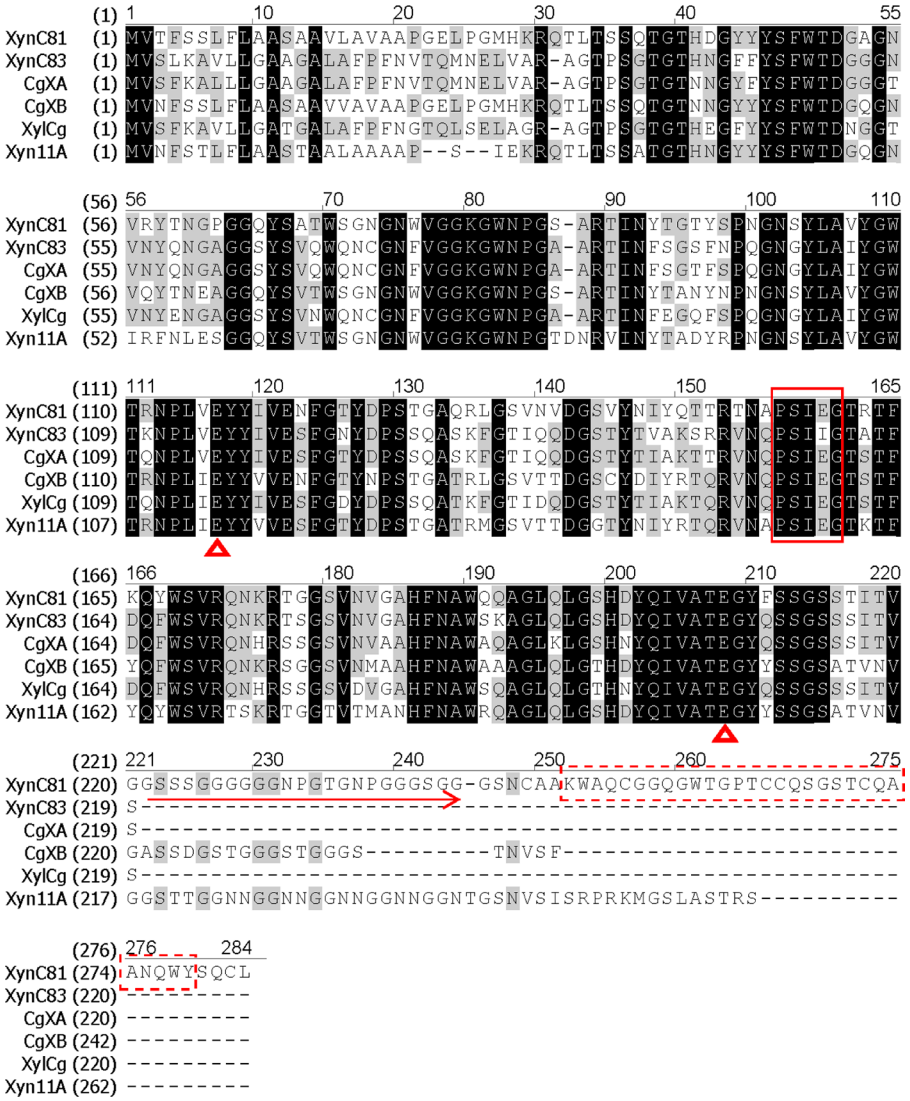

Figure 1. Sequence comparison of XynC81 and XynC83 to other fungal xylanasesL multiple amino acid sequence alignment of xylanases XynC81 and XynC83 from *Achaetomium* sp. Xz-8 with endo-β-1,4-xylanases from *Chaetomium gracile* (CgXA and CgXB; BAA08649.1 and BAA08650.1, respectively) and *Chaetomium globosum* CBS 148.51 (XylCg; EAQ93839.1) and endoxylanase 11A from *Chaetomium thermophilum* (Xyn11A; CAD48749.1). Identical and conserved residues are shaded in black and gray, respectively. The two putative catalytic residues are indicated with triangles. The consensus sequence (PSIXG) of the GH 11 is enclosed in a solid box, and the putative cellulose binding module (CBM) of family 1 and the linker region of XynC81 are indicated by dashed boxes and a horizontal arrow, respectively.

the highest identity of 90% with GH 11 endo-β-1,4-xylanase A (CgXA; BAA08649.1) from *C. gracile* and 65% with the endo-1,4-β-xylanase 2 of GH 11 from *Trichoderma reesei* with crystal structure (PDB 2D97).[24] Both deduced xylanases have two catalytic residues of glutamate and consensus sequence PSIXG (X corresponds to a nonconserved residue) conserved in all GH 11 xylanases (Figure 1). The consensus sequence of the "thumb" loop is of importance in the catalytic efficiency of GH 11 xylanases.[25] On the basis of the template crystal structures (1XNK for XynC81 and 2D97 for XynC83), molecular models of XynC81 and XynC83 were built with Accelrys Discovery Studio software (DS 2.5), respectively (Figure 2). The catalytic domains of XynC81 and XynC83 like other GH 11 xylanases contain two twisted antiparallel β-sheets and a single α-helix that resembles the shape of a partially closed right hand.

Expression and Purification of Recombinant XynC81 and XynC83. The gene fragments coding for mature XynC81 and XynC83 without the signal peptides were cloned into the pPIC9 vector and then transformed into *P. pastoris* GS115 competent cells, respectively. After induction with methanol for 48 h, the highest xylanase activities of 154.5 and 32.2 U/mL were detected in the culture supernatants of recombinant *P. pastoris* harboring *xynC81* and *xynC83*, respectively. The results indicated that both genes encoded functional xylanases and were successfully expressed in *P. pastoris*.

After a three-step purification process, both recombinant XynC81 and XynC83 were purified to electrophoretic homogeneity (Table 2; Figure 3). The specific activity toward beechwood xylan of the pure XynC81 and XynC83 reached 2754.5 and 454.5 U/mg, respectively. Purified XynC81 showed two bands between 26 and 34 kDa on SDS-PAGE, and purified XynC83 showed a single band of approximately 22 kDa. After Endo H treatment specific for N-glycosylation removal, the deglycosylated XynC81 and XynC83 migrated as a single band of about 28 and 22 kDa, respectively, which were essentially identical to their calculated molecular weights. This indicated

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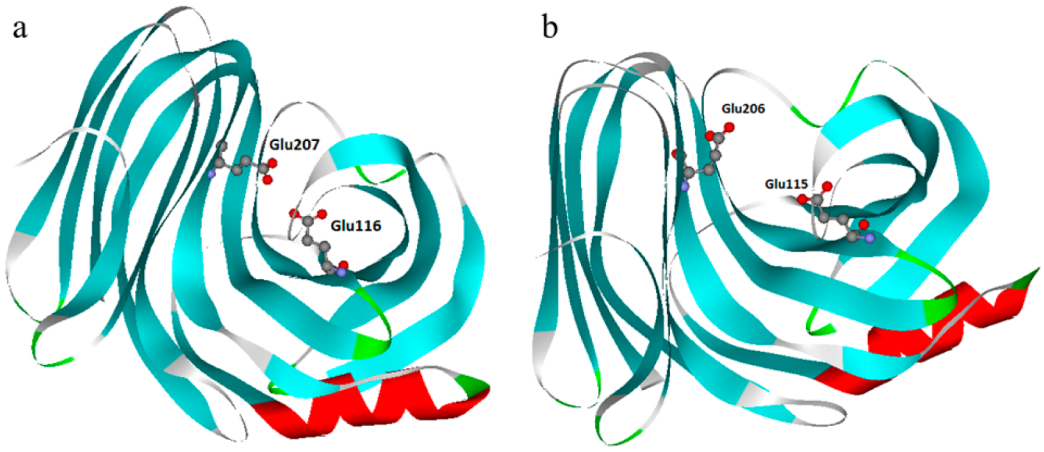

Figure 2. Molecular modeling of XynC81 (a) and XynC83 (b). The α-helix is shown in red, and the β-sheets are in blue. The figures were generated by using Accelrys Discovery Studio software DS 2.5. The putative catalytic site residues, Glu116 and Glu207 of XynC81 and Glu115 and Glu206 of XynC83, correspond to the crystal structures of 1XNK and 2D97, respectively.

Table 2. Summary of the Purification of XynC81 and XynC83

purification step	enzyme activity (U/mL)		total activity (U)		specific activity (U/mg)		purification fold		recovery (%)	
	XynC81	XynC83	XynC81	XynC83	XynC81	XynC83	XynC81	XynC83	XynC81	XynC83
culture supernatant	154.5	32.2	77250	16100	33.6	6.6	1.0	1.0	100.0	100.0
ultrafilteration	1254.8	268.4	62740	13420	94.1	16.5	2.8	2.5	81.2	83.4
cation exchange/gel filtration chromatography[a]	410.4	114.8	10260	2870	2754.5	454.5	82.0	68.9	13.3	17.8

[a]The final purification step was cation-exchange chromatography for XynC81 and gel filtration chromatography for XynC83.

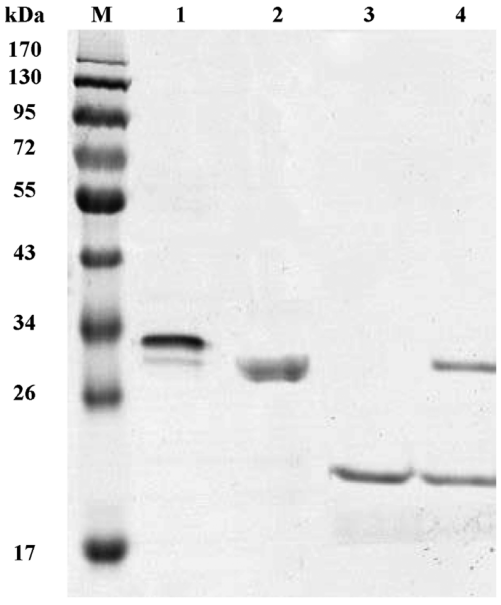

Figure 3. SDS-PAGE analysis of purified recombinant XynC81 and XynC83. Lanes: M, molecular mass standards; 1 and 3, purified recombinant XynC81 and XynC83; 2 and 4, deglycosylated XynC81 and XynC83 with Endo H treatment.

that no glycosylation occurred in XynC83 during heterologous expression in *P. pastoris*.

Enzymatic Properties of Purified XynC81 and XynC83. Purified recombinant XynC81 and XynC83 had a pH optimum at pH 5.5 (at 50 °C), and >60% of their maximum activities was retained between pH 5.0 and 7.0 (Figure 4a). Both enzymes were stable under acidic to alkaline conditions, retaining >75% of the initial activity after preincubation at pH 5.0−11.0 and 37 °C for 1 h (Figure 4b). The optimal temperatures for XynC81 and XynC83 activities were 60−65 °C at pH 5.5 (Figure 4c). The enzymes were stable at 55 °C for 1 h, retaining >80% of their initial activities (Figure 4d). At 65 and 75 °C, both enzymes lost activities rapidly.

The effect of different metal ions or chemical reagents on the activities of purified XynC81 and XynC83 were determined (Table 3). XynC81 was resistant to all tested metal ions and chemical reagents at 1 and 5 mM. XynC83 was resistant to most tested chemicals at low concentration, but was partially inhibited by SDS, Pb^{2+}, Cr^{3+}, Ca^{2+}, Ni^{2+}, and Fe^{3+} at 5 mM concentration. The activities of both XynC81 and XynC83 were enhanced by β-mercaptoethanol.

Kinetic Analysis. According to the Lineweaver−Burk plots (Figure 5), the K_m and V_{max} values of XynC81 were 0.31 ± 0.004 mg/mL and 4049 ± 16.9 μmol/min/mg, respectively, and the K_m and V_{max} values of XynC83 were 0.47 ± 0.007 mg/mL and 848 ± 5.8 μmol/min/mg, respectively. The k_{cat} values of XynC81 and XynC83 were 1866 and 311/s, respectively. The catalytic efficiencies (k_{cat}/K_m) of XynC81 and XynC83 were 6082 and 661 mL/s/mg, respectively.

Substrate Specificity and Analysis of Hydrolysis Products. Purified recombinant XynC81 and XynC83 were most active on soluble wheat arabinoxylan (100%), moderate on beechwood xylan (67 and 69%, respectively) and birchwood xylan (45 and 52%, respectively), and weak on barley β-glucan (18 and 14%, respectively). Only XynC81 had detectable activity against insoluble wheat arabinoxylan (22%). No activity was detected in the presence of CMC-Na and Avicel.

The hydrolysis products of soluble wheat arabinoxylan, beechwood xylan, and birchwood xylan by XynC81 and XynC83 were analyzed by HPAEC (Table 4). Both enzymes were efficient in the hydrolysis of beechwood xylan and

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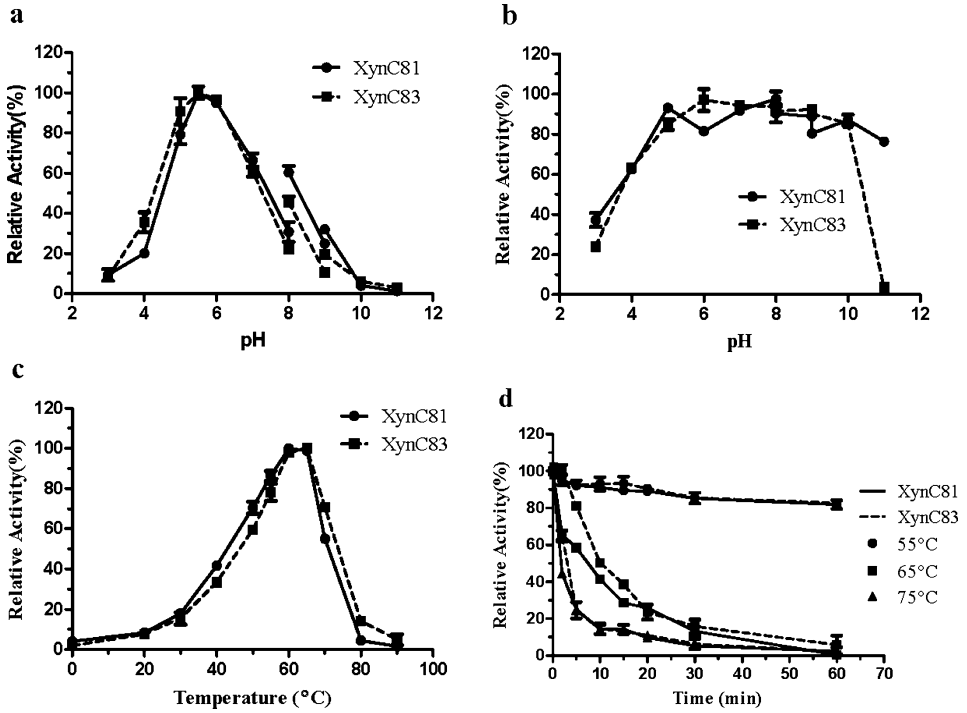

Figure 4. Characterization of purified recombinant XynC81 and XynC83. (a) Effect of pH on enzyme activities. The activity assay was performed at 50 °C in buffers of pH 3.0−11.0 for 10 min. (b) pH stabilities of xylanases at 37 °C for 1 h at pH 3.0−11.0. The residual activity was measured in 100 mM McIlvaine buffer (pH 5.0, 50 °C). (c) Effect of temperature on enzyme activities measured in 100 mM McIlvaine buffer (pH 5.0) for 10 min. (d) Thermostability assay in 100 mM McIlvaine buffer (pH 5.0) at 55, 65, and 75 °C. Each value in the panel represents the mean ± SD (n = 3).

Table 3. Effect of Metal Ions and Chemical Reagents on the Activity of Purified Recombinant XynC81 and XynC83

	XynC81		XynC83	
relative activity[a] (%)	1 mM	5 mM	1 mM	5 mM
control	100.0	100.0	100.0	100.0
Co^{2+}	104.8 ± 1.3	102.1 ± 2.1	97.7 ± 1.3	105.3 ± 0.2
Ni^{2+}	105.2 ± 1.6	99.2 ± 2.4	98.4 ± 1.7	90.1 ± 0.8
Mn^{2+}	101.9 ± 1.6	99.2 ± 0.5	114.0 ± 0.4	99.2 ± 0.9
K^{+}	109.2 ± 1.8	98.5 ± 0.4	121.6 ± 1.6	107.5 ± 0.6
Li^{+}	102.4 ± 1.1	97.2 ± 0.7	118.9 ± 0.2	111.1 ± 0.2
Na^{+}	105.1 ± 0.6	96.9 ± 2.8	121.5 ± 0.4	117.5 ± 2.5
Cu^{2+}	94.5 ± 0.2	88.8 ± 1.8	96.9 ± 1.0	92.0 ± 1.0
Zn^{2+}	100.4 ± 0.3	93.2 ± 1.3	97.8 ± 0.3	100.0 ± 2.1
Pb^{2+}	93.1 ± 2.0	86.6 ± 1.5	100.4 ± 1.0	50.2 ± 0.4
Mg^{2+}	99.8 ± 0.8	92.2 ± 1.6	102.3 ± 0.6	96.6 ± 0.8
Fe^{3+}	95.8 ± 0.3	91.6 ± 0.7	98.2 ± 0.5	89.6 ± 0.7
Ca^{2+}	99.2 ± 0.5	91.6 ± 0.3	92.8 ± 1.1	57.5 ± 1.8
Cr^{3+}	99.4 ± 2.0	89.9 ± 1.0	101.9 ± 0.2	71.5 ± 2.1
β-mercaptoethanol	131.6 ± 0.6	167.0 ± 2.1	133.8 ± 0.3	124.8 ± 1.7
EDTA	100.0 ± 0.8	91.2 ± 0.4	88.3 ± 1.0	68.3 ± 0.5
SDS	89.0 ± 1.3	84.1 ± 1.6	97.8 ± 1.1	72.6 ± 0.6

[a]Values represent the mean ± SD (n = 3) relative to the untreated control samples.

birchwood xylan, approximately 90% of which have been degraded into xylooligosaccharides. The hydrolysis products of soluble wheat arabinoxylan were complex, and the composition of xylooligosaccharides was <40%. Xylobiose and xylotriose were the major hydrolysis products of all tested substrates.

Effects on Filtration Rate and Viscosity of Mash. A total of seven enzyme treatments were used to test their performance in mash processing (Table 5). All of the enzymes tested had positive effects on the filtration rate improvement and viscosity reduction. The highest filtration rate (34.76%) was achieved when the mash was treated with the combination of XynC83 and Glu-66, which was 14.05% higher than the commercial Ultraflo (20.71%). The enzyme combination of XynC81 and XynC83 showed a synergistic effect on viscosity reduction (7.08 vs 6.31% for XynC81 and 5.34% for XynC83), which was comparable to that for Ultraflo (8.47%).

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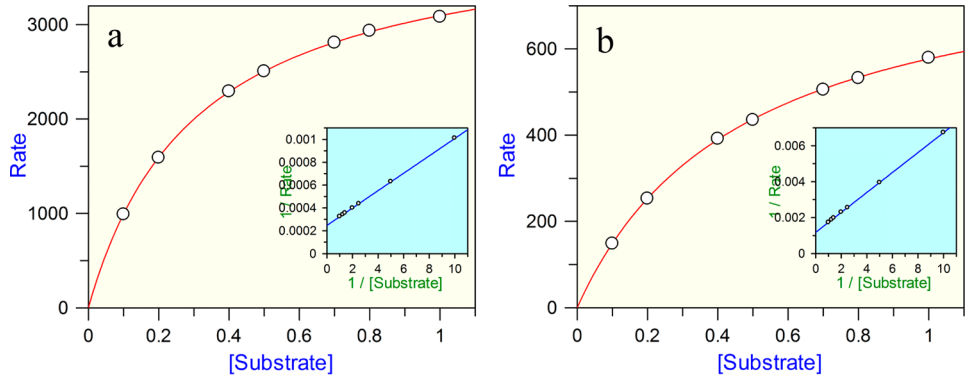

Parameter	Value	Std. Error
Vmax	4048.8028	16.9040
Km	0.3079	0.0036

Parameter	Value	Std. Error
Vmax	848.2277	5.7968
Km	0.4708	0.0073

Figure 5. Lineweaver–Burk plots for the hydrolysis of beech xylan by purified XynC81 (a) and XynC83 (b). The figures were generated by the nonlinear regression computer program GraFit 7.

Table 4. Hydrolysis Products of XynC81 and XynC83

enzyme	substrate	xylose (%)	xylobiose (%)	xylotriose (%)	xylotetraose (%)	undetermined (%)
XynC81	soluble wheat arabinoxylan	5.20	25.12	5.32	0	64.36
	birchwood xylan	2.92	60.99	21.97	3.15	10.97
	beechwood xylan	4.57	61.94	21.76	1.31	10.42
XynC83	soluble wheat arabinoxylan	2.93	27.69	5.51	0	63.87
	birchwood xylan	0	58.77	26.69	3.40	11.14
	beechwood xylan	1.68	60.75	23.83	2.74	11.00

Table 5. Effects of XynC81 and XynC83 Alone or in Combination with Glucanase Glu-66 on the Filtration Rate and Viscosity of Mash

enzyme	filtration rate improvement (%)	viscosity reduction (%)
XynC81	14.29	6.31
XynC83	18.10	5.34
XynC81:XynC83 (1:1)	17.50	7.08
Glu-66	15.51	2.73
XynC81:Glu-66 (1:1)	22.46	6.55
XynC83:Glu-66 (1:1)	34.76	5.68
Ultraflo	20.71	8.47

DISCUSSION

To our knowledge, fungi are generally considered as more potent producers of xylanases than bacteria and yeasts. Fungal genera that are known to produce xylanases include *Aspergillus*, *Disporotrichum*, *Penicillium*, *Neurospora*, *Fusarium*, *Chaetomium*, *Trichoderma*, etc.[6] In this study, we isolated a typical thermophilic fungal strain Xz-8 of *Achaetomium* that showed optimal growth at 45 °C. Two GH 11 xylanase genes (*xynC81* and *xynC83*) were identified in *Achaetomium* sp. Xz-8 and were successfully expressed in *P. pastoris*. This is the first report on gene cloning and expression of xylanases from *Achaetomium*.

Whereas both XynC81 and XynC83 were from the same microbial source and belong to GH 11, they showed only 62% identity to each other. Besides a family 11 catalytic domain, XynC81 contains a C-terminal family 1 CBM. Although they had similar pH and temperature properties, they showed several different characteristics. XynC81showed higher catalytic efficiency than XynC83 and was more resistant to most metal ions and chemical reagents tested. XynC83 could hydrolyze birchwood xylan into a mixture of xylooligosaccharides without xylose. All of these property divergences show the diversity of xylanases from *Achaetomium* sp. Xz-8.

Of all the GH 11 xylanases characterized so far, about 25% of them carry at least one CBM.[2] Unlike the serine/threonine/asparagine-rich linker sequence found in other fungal xylanases, the XynC81 linker sequence is extremely glycine-rich. To our knowledge, CBMs take part in the action of cellulolytic enzymes toward insoluble substrates.[26,27] In the present study, although most enzymatic properties of XynC81 and XynC83 were similar, XynC81 with CBM 1 had activity against insoluble wheat arabinoxylan (22%), whereas XynC83 had not. This result further implied the importance of CBM in enzyme activity toward insoluble substrate.

Most known fungal xylanases of family 11 have acidophilic pH optima, and the temperature optima of GH 11 xylanases from thermophilic fungi are within 62–80 °C.[2] The pH and temperature optima of XynC81 and XynC83 fall within these ranges, exhibiting the highest activity at pH 5.5 and 60–65 °C, respectively. Compared with other GH 11 xylanases with CBM 1, XynC81 has higher optimal temperature and better thermostability.[28−31] The catalytic constant (k_{cat}/K_m) is often recognized as a critical parameter to allow enzyme assessment and comparison. In the present study, XynC81 exhibits a catalytic efficiency of 6082 mL/s/mg, which is the second most efficient xylanase. Compared with other fungal xylanases,

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Table 6. Comparison of the Enzymatic Properties of XynC81 and XynC83 with Those of Other Fungal Counterparts

		optimum					
name	organism	pH	temp	V_{max} (μmol/min/mg)	K_m (mg/mL)	k_{cat} (/s)	k_{cat}/K_m (mL/s/mg)
AnxA[32]	*Aspergillus niger*	5.0	50		4.8	123	26
Xyn11A[28]	*Podospora anserina*	5.0	40	405	5.8	238	41
TfxA[33]	*Thermomonospora fusca*	6.0	60		2.45	139	57
XYNZG[34]	*Plectosphaerella cucumerina*	6.0	40	490	2.1	158	75
PgXynA[35]	*Penicillium griseofulvum*	5.5	55	842	3.3	292	88
XynS20E[36]	*Neocallimastix patriciarum*	5.8	49	153	1.9	185	97
PfXynC[37]	*Penicillium funiculosum*	5.5	55	2835	6.7	997	149
Xyn2[38]	*Trichoderma reesei* Rut C-30	5.0	50		0.114	106	930
PaXyl[39]	*Pholiota adiposa*	5.5	60	6910	3.3	4257	1290
XylCg[40]	*Chaetomium globosum*	5.5	40	4530	0.2	1528	7640
XynC81	*Achaetomium* sp. Xz-8	5.5	60	4049	0.31	1866	6082
XynC83	*Achaetomium* sp. Xz-8	5.5	65	848	0.47	311	661

XynC81 and XynC83 not only have higher catalytic efficiency but also show higher temperature optima (Table 6). The higher catalytic efficiency and optimum temperature of the recombinant xylanases clearly indicate their industrial potential in the hydrolysis of xylan.

The major hydrolysis products of three xylan substrates under study were xylobiose and xylotriose by XynC81 and XynC83. Interestingly, XynC83 hydrolyzed birchwood xylan into a mixture of xylooligosaccharides without xylose. This characteristic has been reported in the GH 11 xylanase from *Streptomyces matensis* DW67[41] and xylanase Xyn802 from *Streptomyces* sp. CS802,[42] which could make XynC83 an ideal candidate for application in xylooligosaccharide production.[43]

In the brewing industry, mashing is a very traditional but complex and remarkably ill-defined process, involving a mix of soluble and insoluble substrates and glycosidase inhibitors. Arabinoxylan is the main nonstarch polysaccharide of barley grain and can cause low extract yield, high wort viscosity, decrease of filtration rate, and formation of some gelatinous precipitates, consequently resulting in a serious technical problem for the brewing industry.[44,45] Considering the conditions of mashing process, such as continuously rising temperature and verified pH values, high-temperature-active xylanases are generally preferred to facilitate the degradation of arabinoxylan in barley malts.[16,46] In this study, XynC81, XynC83, and their combination were supplemented into mash and showed performance in filtration rate improvement and viscosity reduction comparable with that the commercial enzyme complex Ultraflo. However, due to the heterogeneity of the composition and structure of barley, addition of xylanase is insufficient to improve the filtration rate or reduce the viscosity during the mashing process. The effectiveness of the combination xylanase and β-glucanase on barley viscosity decrease could be greater than that of xylanase or β-glucanase alone.[47] In the present study, the noticeable synergistic action of XynC81/XynC83 and the commercial β-glucanase Glu-66 was observed in the filtration rate improvement.

Considering the excellent enzyme properties of XynC81 and XynC83, such as high activity at pH 5.0−7.0 and at 40−70 °C, good thermal and pH stability, less complex hydrolysis products, strong resistance to most metal ions and chemical reagents, and greater catalytic efficiency, XynC83 could be a potential candidate for application in the xylooligosaccharide production, and both XynC81 and XynC83 may represent ideal candidates for application in the brewing industry.

AUTHOR INFORMATION

Corresponding Author

*(B.Y.) Key Laboratory for Feed Biotechnology of the Ministry of Agriculture, Feed Research Institute, Chinese Academy of Agricultural Sciences, No. 12 Zhongguancun South Street, Beijing 100081, P. R. China. E-mail: binyao@caas.cn, yaobin@caas.cn. Phone: +86 10 82106053. Fax: +86 10 82106054.

Author Contributions

‖L.Z. and K.M. contributed equally to this work.

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Notes

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ABBREVIATIONS USED

BLAST, basic local alignment search tool; CBM, carbohydrate binding module; MC-Na, carboxymethyl cellulose sodium; DNS, 3,5-dinitrosalicylic acid; Endo H, endo-β-*N*-acetylglucosaminidase H; FPNI-PCR, fusion primer and nested integrated PCR; GH, glycoside hydrolase; HPAEC, high-performance anion-exchange chromatography; LB, Luria−Bertani; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis

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