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Gene Cloning, Overexpression, and Characterization of a Xylanase from *Penicillium* sp. CGMCC 1669

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Abstract A xylanase-encoding gene, *xyn11F63*, was isolated from *Penicillium* sp. F63 CGMCC1669 using degenerated polymerase chain reaction (PCR) and thermal asymmetric interlaced (TAIL)-PCR techniques. The full-length chromosomal gene consists of 724 bp, including a 73-bp intron, and encodes a 217 amino acid polypeptide. The deduced amino acid sequence of *xyn11F63* shows the highest identity of 70% to the xylanase from *Penicillium* sp. strain 40, which belongs to glycosyl hydrolases family 11. The gene was overexpressed in *Pichia pastoris*, and its activity in the culture medium reached 516 $U ml^{-1}$. After purification to electrophoretic homogeneity, the enzyme showed maximal activity at pH4.5 and 40°C, was stable at acidic buffers of pH4.5–9.0, and was resistant to proteases (proteinase K, trypsin, subtilisin A, and α-chymotrypsin). The specific activity, K_m, and V_{max} for oat spelt xylan substrate was 7,988 $U mg^{-1}$, 22.2 mg ml^{-1}, and 15,105.7 μmol $min^{-1} mg^{-1}$, respectively. These properties make XYN11F63 a potential economical candidate for use in feed and food industrial applications.

Keywords Xylanase · *Penicillium* sp. F63 CGMCC 1669 · *Pichia pastoris* · Overexpression

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Introduction

Xylan is the second most abundant polysaccharide in nature (after cellulose), accounting for approximately one third of all renewable organic carbon on earth [1]. Its complete hydrolysis requires the synergistic action of different xylanolytic enzymes [2, 3]. Among them, endo-1, 4-xylanase (EC3.2.1.8) is the most important enzyme to release long and short xylo-oligosaccharides from various substrates, and thus has been extensively applied in food, textile, animal feed, waste treatment, and paper industries [4].

Xylanases have been reported to be produced by many microorganisms, including fungi, bacteria, and yeasts [5]. Based on the similarities of amino acid sequences, the majority of endo-xylanases fall into glycoside hydrolase (GH) families 10 and 11; whereas, other xylanases are classified into GH families 5, 8, and 43 (http://afmb.cnrs-mrs.fr/CAZY/).

Filamentous fungi are the ideal source of xylanases due to their high xylanase productivity and easy cultivation [6, 7]. To date, several xylanase genes from *Penicillium* spp. have been cloned and expressed, such as *Penicillium citrium* XynA [7], *Penicillium funiculosum* XYNC [8], *Penicillium* sp. strain 40 XynA [9], *Penicillium purpurogenum* XynB [10], and *Penicillium janthinellum* NCIM [11]. However, the use of fungal xylanases is still limited by low yields and high production costs. Therefore, highly desirable xylanases with excellent properties and high yield are still necessary to be identified and explored.

In the present paper, we described the gene cloning, overexpression, and characterization of a GH family 11 xylanase from *Penicillium* sp. F63 CGMCC 1669. The recombinant xylanase was produced in *Pichia pastoris* with high yield and showed high specific activity and strong resistance to proteases, suggesting its potential for industrial applications.

Experimental

Stains, Vectors, Media, and Chemicals

Penicillium sp. F63 CGMCC 1669 was isolated from fermented rapeseed/cottonseed meal (30/70%) and identified using molecular methods. *Escherichia coli* DH5α (TaKaRa, Japan) was cultivated at 37°C in Luria-Bertani medium. *P. pastoris* GS115 (Invitrogen, Carlsbad, CA, USA) was cultivated at 30°C in yeast extract peptone dextrose medium. The plasmids pGEM-T Easy (Promega, Madison, WI, USA) and pPIC9 (Invitrogen) were used for cloning and expression, respectively.

Mandels–Weber's medium [12] for induction of xylanase was composed of (g/l) peptone 0.25, $(NH_4)_2SO_4$ 1.4, KH_2PO_4 2.0, urea 0.3, $CaCl_2$ 0.3, $MgSO_4{\cdot}7H_2O$ 0.3, Tween80 1.0, birchwood xylan 16.3, and 1.0 ml mineral solution (g/l: $FeSO_4{\cdot}7H_2O$ 5.0, $MnSO_4{\cdot}H_2O$ 1.6, $ZnSO_4{\cdot}7H_2O$ 1.4, and $CoCl_2$ 2.0). Buffered glycerol complex medium (BMGY), buffered methanol complex medium (BMMY), regeneration dextrose base medium (RDB), minimal dextrose medium (MD), and minimal methanol medium (MM) were prepared as described in the manual of the Pichia Expression Kit (Invitrogen).

Oat spelt xylan and birchwood xylan were purchased from Sigma (St. Louis, MO, USA). The DNA purification kit, Genome Walking Kit, and *LA Taq* DNA polymerase were purchased from TaKaRa. Restriction endonucleases and *T4* DNA ligase were purchased from New England Biolabs (UK). Other reagents were of analytical grade and commercially available.

Cloning of the Xylanase-encoding Gene *xyn11F63*

Penicillium sp. F63 CGMCC 1669 was grown in Mandels–Weber's medium for 3 days. Mycelia were collected and immediately ground to a fine powder in liquid nitrogen. The genomic DNA and RNA was isolated according to Sambrook et al. [13] and used as the template for polymerase chain reaction (PCR) amplification. The partial core region of the xylanase gene was amplified using degenerate primers GH11F and GH11R (Table 1), which were designed based on the conserved motifs of GH family 11 xylanases. The PCR conditions were as follows: 5 min at 94°C, followed by 34 cycles of 95°C for 30 s, 48°C for 30 s, and 72°C for 1 min. The PCR products were purified and ligated into the pGEM-T Easy vector for sequencing. The nested insertion-specific primers were then designed accordingly (Table 1) and used to amplify the 5′ and 3′ flanking regions of the partial core region. Thermal asymmetric interlaced (TAIL)-PCR [14] was performed with the Genome Walking Kit according to the manufacturer's instructions.

Full-length cDNA of the xylanase was obtained by reverse transcription (RT)-PCR using the total RNA as template. RT reactions were performed using superscript II reverse transcriptase (Invitrogen). PCR amplification was performed using primers xynF and xynR (Table 1) and an annealing temperature of 60°C.

Sequence Analysis

The signal peptide encoding sequence was predicted using SignalP (http://www.cbs.dtu.dk/Services/SignalP). The homology analysis was performed via the Basic Local Alignment Search Tool server. Multiple alignments of protein sequences were accomplished using the ClustalW program (http://www.ebi.ac.uk/clustalW).

Table 1 Primers used in this study.

Primers[a]	Primer sequence (5′→3′)[b]	Size (bp)
GH11F	AACTGCTACCTGGSCITNTAYGGNTGG	27
GH11R	CCGCACGGACCARTAYTGNTIRAANGT	27
xynF (1–35 bp)	ATGGTCTCTTTTTCAAACCTCTTTATGGCTGCCTG	35
xynR (625–654 bp)	TTAGGAAACAGTGATGGACGAAGAGCCACT	30
pic6311F (58–78 bp)	CGGC*GAATTC*CTCCCCAATGAGTTGGATAAG	31
pic6311R (633–654 bp)	ATA*GCGGCCGC*TTAGGAAACAGTGATGGACGAA	33
usp1	GACCACTAGCCCTCTTGTTGAATATTATATCATGGAG	37
usp2	TATCATGGAGAGCTACGGCGAATACAACCC	30
usp3	GCCTTCTATCGTCGCAGATTCCTCCGCT	27
dsp1	GGAGGAATCTGCGACGATAGAAGGCTGG	28
dsp2	CCTGCTTGTGGGTGTAGATATCGTAGACGGAT	32
dsp3	TCTCCATGATATAATATTCAACAAGAGGGCTAGTGGTC	38

[a] Nucleotide position of the oligonucleotides used for amplification reactions in the gene sequence of *xyn11F63*.

[b] S means G or C; Y means C or T; R means A or G; N means A, T, C, or G; I means Hypoxanthine; and restriction sites are in italics

Expression of *xyn11F63* in *P. pastoris*

A gene fragment without the signal peptide encoding sequence was amplified using the primers pic6311F and pic6311R (Table 1, *Eco*RI and *Not*I site contained). The PCR product was cloned into the shuttle vector pPIC9 in-frame fusion with the α-factor signal peptide. The resulting plasmid, pPIC9-*xyn11F63*, was linearized by *Bgl*II and then transformed into *P. pastoris* GS115 competent cells by electroporation. Then, 1 ml of ice-cold 1 mM sorbitol solution was immediately added to the cuvette, mixed completely, spread onto RDB plates, and incubated at 30°C for 72 h. The clones grown on RDB plates were further transferred to MM and MD plates, respectively. After incubation at 30°C for 48 h, the His^+ transformants on MD plates were picked up and incubated in 3 ml of BMGY medium at 30°C for 48 h. The cells were pelleted by centrifugation and re-suspended in 1 ml of BMMY medium. To induce *xyn11F63* expression in *P. pastoris*, methanol was added every 24 h to a final concentration of 0.5%. The cultures were then incubated at 30°C for 60 h with constant agitation. The culture supernatant was collected by centrifugation (12,000 × g for 10 min) for xylanase activity assay. The transformant with highest xylanase activity was cultured in 300 ml of BMGY medium in a 1 l shake flask with agitation at 30°C for 48 h. Then, the cells were harvested and re-suspended in 150 ml of BMMY medium. The culture was incubated at 30°C for 120 h and supplemented with 0.5% methanol every 24 h. The xylanase activity was assayed at an interval of 12 h during induction.

Purification and Mass Spectrometry Analysis of Recombinant XYN11F63

To purify recombinant XYN11F63 (rXYN11F63), the protein in culture supernatant was fractioned by ammonium sulfate (40–80% saturation). The resulting precipitate was dissolved in 20 mM Tris–HCl buffer (pH9.0) and loaded onto a HiTrap Q Sepharose XL 5 ml fast protein liquid chromatography column (Amersham Pharmacia Biotech, Sweden), which was previously equilibrated with the same buffer. Proteins were eluted using a linear gradient of NaCl (0–1.0 M) in the same buffer. Fractions having enzyme activity were pooled and concentrated by ultrafiltration at 4,000 × g for 20 min at 4°C using an Amicon Ultra Centrifugal Filter Device PL-10 (Millipore, Bedford, MA, USA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli [15]. The protein concentration was determined by Bradford assay using bovine serum albumin as the standard [16].

To identify the purified protein, the corresponding band was excised from the SDS-PAGE gel, digested by trypsin under denature conditions, and analyzed using matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry (MALDI-TOF/TOF MS) for peptide finger at Institute of Zoology, Chinese Academy of Sciences.

Xylanase Activity Assay

The xylanase activity was determined by measuring the release of reducing sugar from soluble xylan using the 3,5-dinitrosalicylic acid (DNS) method [17] with xylose as the standard. The standard assay mixtures (total volume 1 ml) contained 0.1 ml of appropriately diluted enzyme sample and 0.9 ml of 1% (*w*/*v*) oat spelt xylan in McIlvaine buffer (0.2 M Na_2HPO_4-0.1 M citric acid, pH4.5). After incubation at 40°C for 10 min, the reaction was terminated by adding 1.5 ml of the DNS reagent. The mixture was then heated in a boiling

water bath for 5 min and cooled down to room temperature, and the absorption at 540 nm was measured. Each reaction and its control were run in triplicate. One unit (U) of xylanase activity was defined as the amount of enzyme that released 1 μmol of reducing sugar equivalent to xylose per minute under the assay conditions.

Characterization of Purified rXYN11F63

The optimum pH of rXYN11F63 for xylanase activity was determined at 40°C in the following buffers of pH 1.0–9.0: 0.1 M KCl-HCl for pH 1.0–2.0, McIlvaine buffer for pH 3.0–8.0, and 0.1 M Tris–HCl for pH 8.0–9.0, and 0.1 M glycine-NaOH for pH 12.0. For the pH stability assay, the purified enzyme was incubated at 37°C in the same buffers mentioned above for 60 min without substrate, and the residual enzyme activity was measured under standard conditions (optimal pH, 40°C for 10 min).

Activity between 0°C and 80°C at optimum pH for 10 min was measured to determine the optimal temperature for rXYN11F63. Temperature stability of the purified recombinant enzyme was determined by measuring the residual enzyme activity under standard conditions after incubation of the enzyme at 40°C, 45°C or 50°C for 0, 2, 5, 10, 15, 20, 30, 45, or 60 min.

To determine resistance to proteolysis, purified rXYN11F63 was incubated with either trypsin or alpha-chymotrypsin in 0.1 M Tris–HCl (pH 7.0), or proteinase K or subtilisin A in 0.1 M Tris–HCl (pH 7.5) at 37°C for different periods (30 or 60 min) at a ratio of 0.1:1 (protease:rXYN11F63, *w*/*w*). Protease resistance was assessed by measuring the residual enzyme activity under standard conditions following protease treatment.

The effect of metal ions and chemical reagents on the activity of purified rXYN11F63 was determined by adding 1, 5, or 10 mM of various metals (NaCl, KCl, $CaCl_2$, LiCl, $CoCl_2$, $CrCl_3$, $NiSO_4$, $CuSO_4$, $MgSO_4$, $FeCl_3$, $MnSO_4$, $ZnSO_4$, Pb $(CH_3COO)_2$, $AgNO_3$, or $HgCl_2$) or reagents (SDS, ethylenediaminetetraacetic acid, or β-mercaptoethanol) to the enzyme assay system. The system without any additive was used as a control.

Substrate Specificity and Kinetic Parameters

The substrate specificity of rXYN11F63 was determined by measuring the enzyme activity after incubation in McIlvaine buffer (pH 4.5) containing 0.5% (*w*/*v*) of each substrate (oat spelt xylan, birchwood xylan, lichenan, barley glucan, and CMC-Na) at pH 4.5 and 40°C for 10 min. Kinetic parameters, V_{max}, K_m, and k_{cat}, were determined in McIlvaine buffer (pH 4.5) containing 1.0–10 mg ml^{-1} substrates after incubation with purified rXYN11F63 at pH 4.5 and 40°C for 5 min. The data were plotted according to the Lineweaver–Burk method. Each data was an average of three independent experiments, and every experiment included three samples.

Analysis of Hydrolysis Products

The reaction mixture containing 100 U purified recombinant enzyme and 1 mg xylan (from oat spelt and birchwood) in 200 μl McIlvaine buffer (pH 4.5) was incubated at 37°C for 12 h. After hydrolysis, the enzyme was removed from the reaction system using the Nanosep Centrifugal 3 K Device (Pall, Ann Arbor, MI, USA). The products were analyzed by high-performance anion-exchange chromatography (HPAEC) with a model 2500 system from Dionex (Sunnyvale, CA, USA) [18]. Xylose, xylobiose, xylotriose, and xylotetraose

were used as standards. The hydrolytic products were quantified on the basis of their own standard curves.

Nucleotide Sequence Accession Numbers

The nucleotide sequences for the *Penicillium* sp. F63 CGMCC1669 18S rDNA and the xylanase gene (*xyn11F63*) were deposited in the GenBank database under accession numbers DQ499658 and FJ899681, respectively.

Results

Strain Identification and Xylanase Activity

The PCR-amplified partial 18S rDNA sequence (1,201 bp) of strain F63 revealed the highest nucleotide identity (98%) with that from *Penicillium* spp. (GenBank accession no. AJ005446.1 and FJ430772.1), thus, strain F63 was classified into the genus *Penicillium* and deposited in the China General Microbiological Culture Collection Center (CGMCC) under CGMCC 1669. The xylanase activity of the native strain was 20.2 Uml^{-1} after xylan induction for 12 h.

Gene Cloning of *xyn11F63* and Sequence Analysis

Amino acid sequence alignment of known GH 11 xylanases revealed two highly conserved motifs, S-Y-L[C/S/A]-[V/L]-Y-G-W and T-F-[V/L]-Q-[Y/F]-[W/F]-S-V, and a degenerate primer set (GH11F and GH11R) was designed accordingly (Table 1). Using this primer set, a partial core region fragment of xylanase gene (213 bp) was amplified, and the PCR product was cloned into pGEM-T Easy and sequenced. The 5′ and 3′ flanking sequences of the partial core region were then amplified by TAIL-PCR using nested insertion primers. Two PCR products from the 5′ and 3′ flanking regions, about 767 and 1,321 bp, respectively, were obtained and assembled with the core region. The resulting DNA sequence was 2,301 bp. One complete open reading frame consisting of 724 bp was identified.

The full-length cDNA sequence of the xylanase gene (*xyn11F63*), 651 bp in length, was obtained by RT-PCR using the specific primers xynF and xynR (Table 1). One 73-bp intron interrupts the xylanase-encoding sequence. SignalP analysis revealed the existence of an *N*-terminal signal peptide at residues 1–19. The mature protein is 198 amino acid residues with a theoretical molecular weight of 21.5 kDa.

The deduced amino acid sequence of *xyn11F63* showed the highest identity of 85% to a putative xylanase from *Penicillium chrysogenum* (BAG75460) and of 70% to XynA from *Penicillium* sp. strain 40 (BAA88421) [9], both of which belong to GH family 11. The structure-based sequence alignment between XYN11F63 and XynC from *Aspergillus kawachii* (protein data bank (PDB) code 1bk1, which showed 47% of amino acid sequence identity to XYN11F63) was carried out (Fig. 1). XYN11F63 showed the similar protein structure as *A. kawachii* XynC did. Two conserved catalytic residues, Glu-112 and Glu-204, were found in the amino acid sequence of XYN11F63. No carbohydrate-binding domain and potential *N*-glycosylation sites (Asn-X-Ser/Thr, in which X represents any amino acid other than praline) were identified in the protein sequence [19].

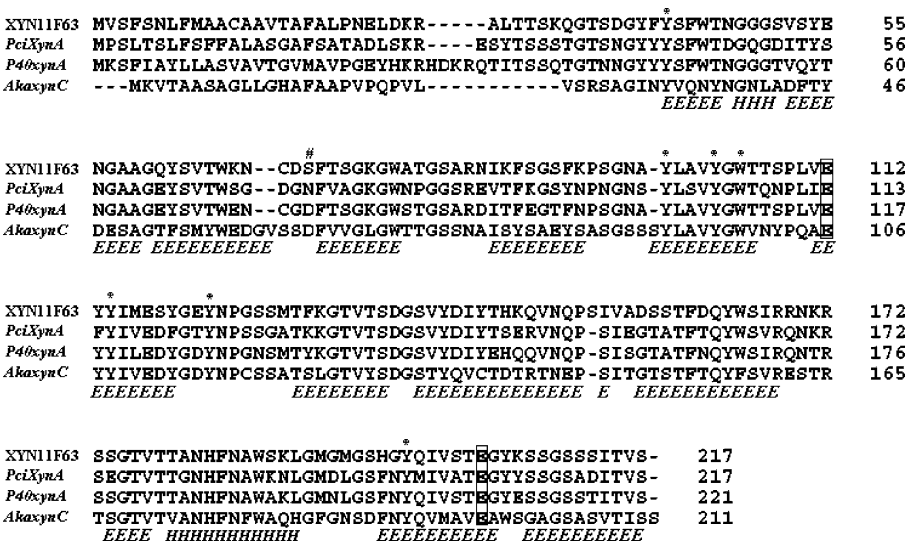

Fig. 1 Structure-based sequence alignment of XYN11F63 with related amino acid sequences of xylanases with known structures. The alignment was carried out by ClustalX. The catalytic residues are *boxed*, and conserved residues are marked with *asterisks*. The residues marked *E* indicate beta strands; the residues marked *H* indicate alpha helices. The structure elements were analyzed with the xylanase sequence of *Aspergillus kawachii* (protein data bank code 1bk1) using the DALI program (http://ekhidna.biocenter.helsinki.fi/dali_server/)

Expression of *xyn11F63* in *P. pastoris*

The gene fragment encoding the mature xylanase was amplified by PCR and cloned into a pPIC9 vector to form a recombinant expression vector pPIC9-*xyn11F63*. After linearization by *Bgl*II digestion, pPIC9-*xyn11F63* was transformed and integrated into *P. pastoris* GS115. Positive transformants were screened based on the xylanase activity. The highest xylanase activity in the culture supernatant of 1 l shaker flask was 516 Uml^{-1} after induction with methanol for 72 h, confirming that *xyn11F63* encoding a functional xylanase have been successfully expressed in *P. pastoris.* The recombinant protein showed a band of about 21 kDa when subjected to SDS-PAGE analysis (Fig. 2), which was consistent with the predicted molecular weight. No activity or protein band was detected in the culture of transformants harboring empty vector.

Purification and Mass Spectrographic Analysis of rXYN11F63

rXYN11F63 was purified by ammonium sulfate precipitation and anion-exchange chromatography. The specific activity of rXYN11F63 was 7,988 Umg^{-1} after 2.33-fold purification with a final yield of 22.9%. The purified rXYN11F63 migrated as a single band of about 21 kDa on the SDS-PAGE gel (Fig. 2). By using MALDI-TOF/TOF MS, six peptides, NCDSFTSGK, GTVTSDGSVYDIYTHK, QVNQPSIVADSSTFDQYWSIR, RSSGTVTTANHFNAWSKLG, MGMGSHGYQI, and VSTEGYK, were identified in the purified protein. These amino acid sequences shared 100% identity with the predicted sequence of XYN11F63, indicating the purified protein was the exact recombinant xylanase.

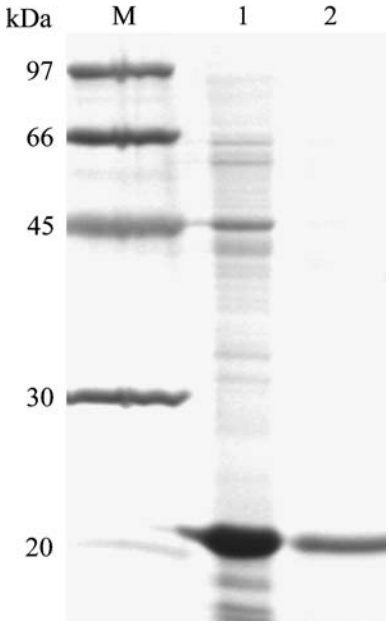

Fig. 2 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the recombinant XYN11F63 expressed in *Pichia pastoris*. Lanes *M*, low molecular weight marker; *1*, culture supernatant of the induced transformant harboring pPIC9-*xyn11F63*; *2*, rXYN11F63 after purification

Properties of rXYN11F63

Purified rXYN11F63 exhibited optimal enzyme activity at pH4.5 and retained more than 55% of the maximum activity at pH3.5–5.5 (Fig. 3a). The enzyme was stable over pH4.5–9.0, retaining more than 90% of the maximum activity after incubation at 37°C for 60 min (Fig. 3b). The optimal temperature for xylanase activity at pH4.5 was 40°C (Fig. 3c), and more than 60% of the maximum activity was remained when assayed at 30–50°C. Purified rXYN11F63 retained 91.7% and 44.6% of its maximal activity after pre-incubation at 40°C and 45°C for 60 min, respectively (Fig. 3d). After incubation at 50°C for 30 min, the enzyme lost almost all of the activity.

After treatment with trypsin, α-chymotrypsin, proteinase K, and subtilisin A at 37°C for 30 min, the recombinant enzyme retained 92.7%, 93.1%, 81.0%, and 86.8% of its activity, respectively. When treated for 60 min, more than 80% of the activity remained (Fig. 3e).

The xylanase activity of purified rXYN11F63 in the presence of different metal ions or chemical reagents is shown in Table 2. Enzyme activity was not significantly affected by most metal ions and reagents at the concentration of 1 mM. The activity was almost completely inhibited by Hg^{2+} and strongly inhibited by SDS. Partial inhibition was observed in the presence of some metal ions and reagents, such as Co^{2+}, Cu^{2+}, Pb^{2+}, and Fe^{3+}, at 10 mM concentration. β-mercaptoethanol at 5 mM and 10 mM significantly enhanced the activity about 1.29- and 1.35-folds, respectively.

Substrate Specificity and Kinetic Parameters of XYN11F63

Purified rXYN11F63 exhibited high activity on substrate oat spelt xylan (100%) and birchwood xylan (64.2%) but had no activity towards CMC-Na, lichenan, or barley β-glucan. The V_{max}, K_m, and k_{cat} values were 15,106 μmol $min^{-1} mg^{-1}$, 22.2 mg ml^{-1}, and

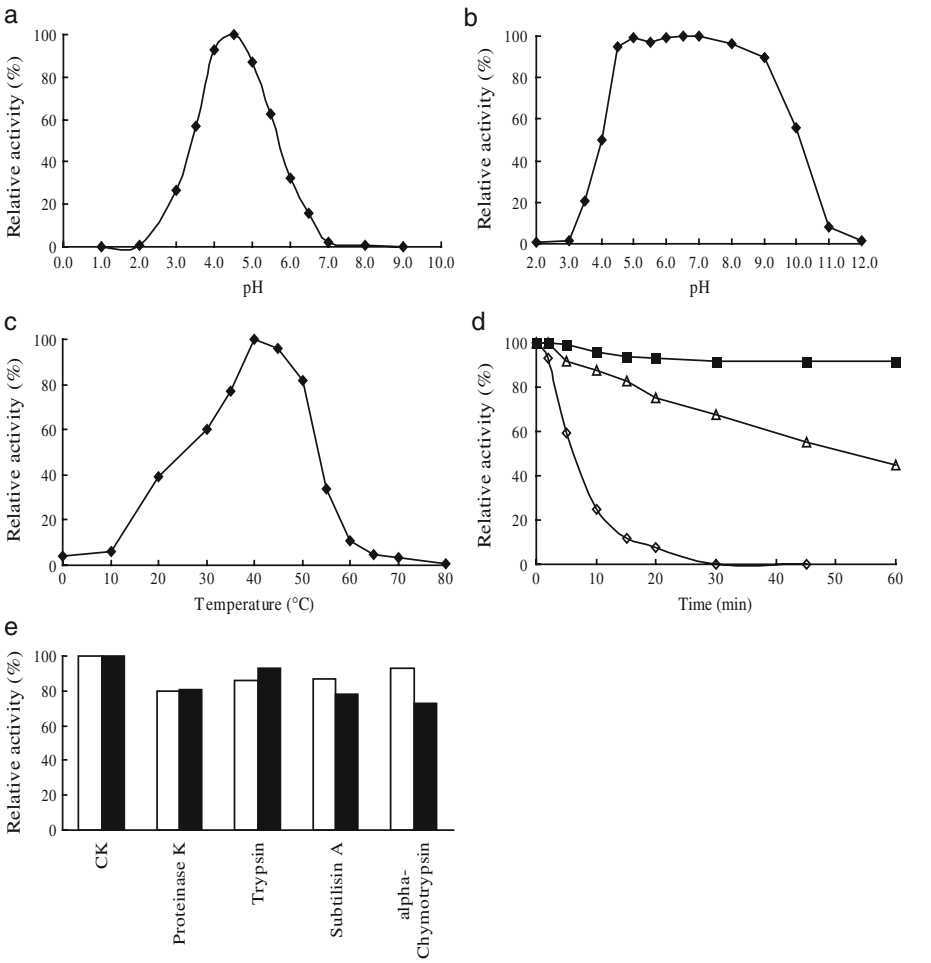

Fig. 3 Properties of the purified rXYN11F63. **a** Effect of pH on xylanase activity. The assay was performed at 40°C in buffers with pH ranging from 1.0 to 9.0. **b** pH stability of rXYN11F63. After incubating the enzyme at 37°C for 1 h in buffers of pH2.0–12.0, the activity was measured in McIlvaine buffer (pH4.5) at 40°C. **c** Effect of temperature on xylanase activity measured in McIlvaine buffer (pH4.5). **d** Temperature stability of rXYN11F63. The enzyme was pre-incubated at 40 (*filled square*), 45 (*open triangle*), and 50°C (*open diamond*) in McIlvaine buffer (pH4.5), and aliquots were removed at specific time points for the measurement of residual activity at 40°C. **e** Effect of proteases on xylanase activity. The residue activity was determined after treatment with proteases for 30 (*open square*) or 60 (*closed square*) min. *CK* indicates no proteases was added

5,416 s^{-1} for oat spelt xylan, respectively, and 10,571 μmol $min^{-1}mg^{-1}$, 11.3 mg ml^{-1}, and 3,790 s^{-1} for birchwood xylan, respectively.

Analysis of Hydrolysis Products

The products of rXYN11F63 hydrolysis on oat spelt xylan and birchwood xylan were different based on the HPAEC analysis. The main hydrolysis products of oat spelt xylan

Table 2 Effect of metal ions and chemical reagents on the activity of rXYN11F63.

Chemicals	Relative activity (%)[a]			Chemicals	Relative activity (%)		
	1 mM	5 mM	10 mM		1 mM	5 mM	10 mM
None	100.0	100.0	100.0	Zn^{2+}	97.5	97.0	85.4
Na^{+}	101.5	102.8	103.0	Pb^{2+}	86.8	77.4	57.9
K^{+}	106.4	103.8	104.2	Fe^{3+}	89.7	83.2	60.3
Li^{+}	101.2	91.9	92.8	Mn^{2+}	91.6	–	–
Ca^{2+}	109.8	88.0	92.3	Ag^{+}	94.3	10.6	–
Mg^{2+}	99.9	97.2	94.9	EDTA	92.3	90.3	87.1
Co^{2+}	96.7	86.0	76.2	Hg^{2+}	4.0	0.0	0.0
Cr^{3+}	99.9	93.9	82.9	SDS	45.8	11.8	8.9
Cu^{2+}	88.1	62.1	52.3	β-Mercaptoethanol	117.1	129.2	134.6
Ni^{2+}	99.6	90.6	88.5				

[a] Values represent the means of triplicates relative to the untreated control samples.

were 7.46% xylose, 28.36% xylobiose, 25.37% xylotriose, and 38.81% xylotetraose. The mass composition of the hydrolysis products from birchwood xylan was 11.76% xylose, 43.53% xylobiose, 44.71% xylotriose, and no xylotetraose.

Discussion

In the point of industrial application, filamentous fungi are the most excellent sources of xylanases because of their high productivity [6]. *Penicillium* is mostly saprophytic in nature, and numerous species are of particular value for humanity. The production of xylanolytic enzymes by *Penicillium* has been explored in some species [20]. An extracellular GH family 36 α-galactosidase from *Penicillium* sp. F63 has been reported [21]. In the present work, xylanase activity of *Penicillium* sp. F63 (20.2 Uml^{-1}) was detected after induction with birchwood xylan. Except for the xylanase-encoding gene—*xyn11F63*, another xylanase gene encoding an intracellular GH family 10 xylanase was also cloned (data not shown). The high native xylanase activity might be due to the existence of two or more xylanase genes in *Penicillium* sp. F63, although the activity was not high enough for direct industrial production.

In order to further reduce production costs, high-level expression of xylanases or exploring xylanases with high specific activity is generally preferred. In this study, *xyn11F63* was overexpressed in *P. pastoris* with activity of up to 516 Uml^{-1} in the shake flask culture. The specific activity of the purified XYN11F63 was 7,988 Umg^{-1}, significantly higher than that of most *Penicillium* xylanases, such as XYNA from *Penicillium* sp. strain 40 (1,250 Umg^{-1}) [9], XynA from *P. citrinum* (2,100 Umg^{-1}) [7], and XYNC from *P. funiculosum* (2,830 Umg^{-1}) [8]. The high level of expression would be further improved by optimizing the fermentation parameters. Any way, the high expression and high specific activity of rXYN11F63 make it more economical in xylanase production.

Purified rXYN11F63 had pH optimum of 4.5 and remained active over pH2.0–7.0, indicating that rXYN11F63 was weak acidic, the same as those of GH family 11 xylanases from *Penicillium sclerotiorum* [20] and *Penicillium capsularium* [22]. Some fungal xylanases have acidophilic features with pH optima of 2.0–4.0, such as *Penicillium* sp.

strain 40 XynA [9], *A. kawachii* XynC [23], and *Aspergillus niger* XynI [24]. Some *Penicillium* xylanases have higher pH optima. For example, *P. citrium* XynA showed optimal activity at pH5.0 [7]. The three-dimensional model and mutation analysis of *A. kawachii* XynC [23] have shown that residues Asp and Glu locating at the upper and lower edges of the active site cleft, respectively, played an important role in its low pH optimum. It was proposed that Asp can be protonated, and the proton of catalytic Glu is available for catalysis at low pH [25]. The corresponding residues in rXYN11F63 were Ser-71 (Fig. 1, marked by #) and Gln-151, and Asn/Gln in *P. citrium* XynA, both of which could not be protonated at acidophilic buffers. The mechanisms of acid stability and acidophilicity enable us to further engineer the enzymes with different pH optima.

rXYN11F63 showed excellent substrate specificity. It can hydrolyze commercial xylans and has no activity towards glucan, CMC-Na, and lichenan. Based on the HPAEC analysis, the hydrolysis products to birchwood xylan and oat spelt xylan were different. Both substrates have the following components in the hydrolysis products: xylose, xylobiose, and xylotriose; and xylotetraose was only detected in the hydrolysis products of oat spelt xylan. This difference probably ascribed to the xylan composition: birchwood xylan contains more than 90% of xylose residues while less than 80% in oat spelt xylan [26]. The more branched other oligosaccharides may lead to the production of xylotetraose. However, the produced oligosaccharides may be beneficial for animal health. Therefore, combining with its acidic pH optimum and strong protease resistance, rXYN11F63 may facilitate its potential application in feed and food industries.

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