Appl Biochem Biotechnol (2010) 162:953–965
DOI 10.1007/s12010-009-8815-5

Gene Cloning, Expression, and Characterization of a Thermostable Xylanase from *Nesterenkonia xinjiangensis* CCTCC AA001025

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Received: 29 July 2009 / Accepted: 7 October 2009 /
Published online: 18 October 2009

Abstract An endo-β-1,4-xylanase-encoding gene, *xyn11NX*, was cloned from *Nesterenkonia xinjiangensis* CCTCC AA001025 and expressed in *Escherichia coli*. The gene encoded a 192-amino acid polypeptide and a putative 50-amino acid signal peptide. The deduced amino acid sequence exhibited a high degree of similarity with the xylanases from *Streptomyces thermocyaneoviolaceus* (68%) and *Thermobifida fusca* (66%) belonging to glycoside hydrolase family 11. After purification to homogeneity, the recombinant Xyn11NX exhibited optimal activity at pH 7.0 and 55 °C and remained stable at weakly acidic to alkaline pH (pH 5.0–11.0). The enzyme was thermostable, retaining more than 80% of the initial activity after incubation at 60 °C for 1 h and more than 40% of the activity at 90 °C for 15 min. The K_m and V_{max} values for oat spelt xylan and birchwood xylan were 16.08 mg ml^{-1} and 45.66 μmol min^{-1} mg^{-1} and 9.22 mg ml^{-1} and 16.05 μmol min^{-1} mg^{-1}, respectively. The predominant hydrolysis products were xylobiose and xylotriose when using oat spelt xylan or birchwood xylan as substrate.

Keywords *Nesterenkonia xinjiangensis* · Xylanase · Thermostability · Glycoside hydrolase (GH) family 11

Introduction

Xylan is the major component of plant hemicellulose and represents the second most abundant renewable polysaccharide in nature [1]. It is a heterogeneous polysaccharide

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consisting of a main chain of β-1,4-linked-D-xylose residues that often carry arabinosyl, acetyl, and glucuronosyl substituents [2, 3]. The complete hydrolysis of xylan requires the cooperative action of several enzymes such as endoxylanase, β-xylosidase, α-arabinosidase, and acetyl esterase. Among them, endo-β-1,4-xylanase (EC 3.2.1.8) is a crucial enzyme by catalyzing the degradation of xylan into short xylooligosaccharides [2].

Based on structural similarity among catalytic domains, majority of xylanases fall into glycoside hydrolase (GH) family 10 and 11 [4]. Xylanases belonging to family 11 have no cellulase activity and are smaller (around 20 kDa) than those belonging to family 10 xylanases (more than 40 kDa; with cellulase activity). The smaller size and compact folding of family 11 xylanases enable them to easily penetrate the cellulose fiber network without damaging the fiber; thus these enzymes are suitable in bioleaching processes [5, 6].

Microbial xylanases have attracted considerable interest because of their potential applications in the food, animal feed, and paper and pulp industries [7–9]. Such applications generally require a thermostable and cellulase-free xylanase with broad pH and temperature adaptability [7, 9]. Pulp and paper industry is a primary target for the potential use of xylanases since replacing chlorine with xylanase during pulp processing not only has environmental benefits but is also cost-effective [7–9].

In this paper, we reported a new family 11 xylanase-encoding gene from *Nesterenkonia xinjiangensis*. The distinctive characteristics of this strain are its ability to grow over a broad alkaline pH range (pH 7.0–12.0) and to produce extracellular xylanase in the presence of xylan as substrate. Thus, *N. xinjiangensis* was chosen for cloning and expression of xylanase gene. The recombinant enzyme was purified, characterized, and showed good thermostability and cellulase-free activity. It is the first report on cloning and expression of a thermostable xylanase from the genus *Nesterenkonia*.

Materials and Methods

Enzymes and Chemicals

Restriction endonucleases and DNA purification kit were purchased from TaKaRa (Dalian, China). The low molecular weight electrophoresis calibration kit was supplied by Amersham Bioscience (New Territories, HK, China). The protein assay kit was obtained from Bio-Rad (Hercules, CA, USA). T4 DNA ligase was purchased from New England Biolabs (Beverly, MA, USA). All other chemicals used were of analytical grade unless otherwise stated and commercially available.

Strains and Vectors

N. xinjiangensis CCTCC AA001025 was purchased from the China Center for Type Culture Collection. This strain was able to produce extracellular xylanase on oat spelt xylan and showed xylanolytic activity of 2.63 U ml^{-1} at pH 6.0 and 1.92 U ml^{-1} at pH 9.0, respectively. *Escherichia coli* Top10 (TransGen, Beijing, China) and *E. coli* BL21 (DE3) (TaKaRa, Ostu, Japan) were used for gene cloning and expression, respectively. The vectors pGEM-T easy (Promega, Madison, WI, USA) and pET-22b(+) (Novagen, San Diego, CA, USA) were utilized for sequencing and recombinant expression plasmid construction, respectively.

Gene Cloning

The xylanase gene (*xyn11NX*) of *N. xinjiangensis* was cloned using a two-step PCR approach—a touchdown PCR step and a TAIL-PCR step. The degenerate primer set specific for family 11 xylanases from *Streptomyces* [10] was used in the touchdown PCR. The genomic DNA of *N. xinjiangensis* CCTCC AA001025 was extracted and used as template for amplification. The PCR conditions were as follows: 5 min at 95 °C, followed by 11 cycles of 95 °C for 30 s, 50–45 °C (decreasing by 0.5 °C after each cycle) for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 7 min. The amplified fragment of appropriate size (about 500 bp) was ligated into the pGEM-T easy vector for sequencing and BLAST analysis. The 5′ and 3′ flanking regions of the core region were obtained using TAIL-PCR with six nested insertion-specific primers (usp1-3 and dsp1-3; Table 1). The TAIL-PCR reaction was performed with the Genome Walking kit (TaKaRa) following the manufacturer's instructions.

Sequence Analysis

The sequence assembly was performed using programs from Vector NTI Advance 10.0 software (Invitrogen, Carlsbad, CA, USA), and the nucleotide sequence was analyzed using the NCBI ORF Finder tool (http://www.ncbi.him.nih.gov/gorf/gorf.html). The presence of signal peptide in the deduced amino acid sequence was predicted using SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/). The DNA and protein sequence alignments were carried out using the blastn and blastp programs (http://www.ncbi.nlm.nih.gov/BLAST/), respectively. Multiple alignments of the protein sequences were performed using the ClustalW program (http://www.ebi.ac.uk/clustalW/). The protein structure model was predicted by SWISS-MODEL (http://swissmodel.expasy.org//SWISS-MODEL.html) with the *Chaetomium thermophilum* xylanase (Protein data bank code, 1H1A_A) as the template.

Expression of Xyn11NX in *E. coli*

To construct the expression vector, a gene fragment coding for the mature xylanase was amplified from the genomic DNA of *N. xinjiangensis* using the LA Taq DNA polymerase

Table 1 Primers used in this study.

Primer name	Primer sequence[a]	Size (bp)	References
S11F	5′-TACTCSTTCTGGACSGAC-3′	18	[18]
S11R	5′-CTGCTCTGRTANCCYTC-3′	17	[18]
usp1	5′-GCTGCCGTCCGACCAGAAGGTG-3′	22	This study
usp2	5′-GGTCCATCCGTAGAGGGCCAGGTAG-3′	25	This study
usp3	5′-CCACGAAGTTCCCGGTGTCTCC-3′	22	This study
dsp1	5′-ACGGCACCTACCGTCCCACCG-3′	21	This study
dsp2	5′-CGAGACCACTCGGGTCGAGGAGC-3′	23	This study
dsp3	5′-CGATCACCACGGCGAACCACTTC-3′	23	This study
xyn11NXEF	5′-CCG***GAATTC***GCAGCCGGTCTACGAGGACACCC-3′	32	This study
xyn11NXER	5′-ATAAGAAT***GCGGCCGC***CGGGGCGGTGTGGACCGTCACC-3′	38	This study

[a] Restriction enzyme sites incorporated into the primers are shown in bolded italic type

with GC Buffer I (TaKaRa) and primers xyn11NXEF and xyn11NXER (Table 1). The PCR reaction consisted of an initial step of 5 min at 95 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 70 °C, and 1 min at 72 °C, and a final extension step of 7 min at 72 °C. The PCR product was gel-purified, digested with *Eco*RI and *Not*I, and then cloned into the corresponding sites of pET-22b(+). The recombinant plasmid pET-*xyn11NX* was transformed into *E. coli* BL21 (DE3) competent cells. Several positive transformants were cultured in 20 ml LB medium containing 100 μmol ml^{-1} ampicillin at 37 °C to an OD_{600} of 0.6–0.8. Xylanase expression was induced by addition of 1 mM IPTG for an additional 16 h at 20 °C. Protein expression in the culture supernatant was checked by activity assay and SDS-polyacrylamide gel electrophoresis (SDS-PAGE). *E. coli* BL21 (DE3) harboring empty plasmid and recombinant *E. coli* BL21 (DE3) without induction were treated as negative controls.

Purification of Recombinant Xyn11NX

The induced culture (1,000 ml) was centrifuged at 12,000×*g*, 4 °C for 10 min to remove cell debris. The cell-free supernatant was concentrated to 30 ml with a hollow fiber membrane with a molecular weight cut-off of 6 kDa (Motimo, Tianjin, China) and a Vivaflow 200 system (Vivascience, Hanover, Germany). The crude enzyme was purified by Ni-NTA affinity chromatography using a HisTrap HP column (1.0 ml; GE Healthcare, Uppsala, Sweden) and eluted using an imidazole step gradient (0, 20, 40, 60, 80, 100, 200, and 300 mM) in 20 mM Tris–HCl (pH 7.6) containing 500 mM NaCl and 10% (*w*/*v*) glycerol. Protein purity was verified by SDS-PAGE analysis. The protein concentration of the purified recombinant Xyn11NX was determined by Bradford method [11] using bovine serum albumin as a standard. The identity of the purified enzyme was determined by liquid chromatography–electrospray ionization–tandem mass spectrometry (LC–ESI–MS/MS), and then the resulting peptide sequences were compared with the known amino acid sequence of recombinant Xyn11NX.

Enzyme Assay

Xylanase activity was determined by measuring the amount of reducing sugar released from oat spelt xylan using a 3,5-dinitrosalicylic acid (DNS) reagent as described by Miller [12]. The reaction mixture containing 0.9 ml of 1.0% (*w*/*v*) oat spelt xylan and 0.1 ml of suitably diluted enzyme was incubated in McIlvaine buffer (0.2 M Na_2HPO_4/0.1 M citric acid, pH 7.0) at 55 °C for 10 min. The reaction was stopped by adding 1.5 ml of 1.0% (*w*/*v*) DNS. The mixture was then boiled for 5 min and cooled, and the optical density was measured at 540 nm. 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. Specific activities were expressed as units per milligram of protein. All the experiments were performed in triplicates.

Biochemical Characterization

The pH profile of purified recombinant Xyn11NX was determined by measuring the xylanase activity at 55 °C in buffers of varying pH ranging from 2.0 to 12.0. The buffers used were McIlvaine buffer for pH 2.0–8.0, 0.1 M Tris–HCl for pH 8.0–9.0, and 0.1 M glycine-NaOH for pH 9.0–12.0. The effect of pH on enzyme stability was estimated by pre-incubating the purified enzyme in different buffers of pH 3.0–12.0 at 37 °C for 1 h and then measuring the residual enzyme activity under standard conditions.

The optimum temperature was determined at the optimum pH by varying the temperature from 20 to 70 °C. The thermostability of the enzyme was assessed by determining the residual enzyme activity after incubation of the enzyme in McIlavaine buffer with optimum pre-determined pH at 60, 70, 80, and 90 °C for designated time periods.

The effect of metal ions and chemical reagents on the activity of the purified enzyme was investigated by measuring the xylanase activity in the presence of 1, 5, or 10 mM Na^+, K^+, Ca^{2+}, Li^+, Co^{2+}, Cr^{3+}, Ni^{2+}, Cu^{2+}, Mg^{2+}, Fe^{2+}, Mn^{2+}, Zn^{2+}, Pb^{2+}, Ag^+, Hg^{2+}, SDS, EDTA, or β-mercaptoethanol. The test system without addition of ions or reagents was used as control.

In order to examine its resistance to different proteases, the purified recombinant enzyme (100 μg ml^{-1}) was incubated with 10 μg ml^{-1} trypsin (from bovine, pH 7.6, 25 °C, 14,700 U mg^{-1}; Sigma), 250 μg ml^{-1} α-chymotrypsin (type II from bovine, pH 7.8, 25 °C, ≥40 U mg^{-1}; Sigma), 20 μg ml^{-1} collagenase (type IV from *Clostridium histolyticum*, pH 7.4, 37 °C, 527 U mg^{-1}; Sigma), 500 μg ml^{-1} subtilisin A (type VIII from *Bacillus licheniformis*, pH 7.5, 37 °C, 10 U mg^{-1}; Sigma), 330 μg ml^{-1} proteinase K (type VIII from *B. licheniformis*, pH 7.5, 37 °C, 30 U mg^{-1}; Amresco, Solon, USA), 10 mg ml^{-1} proleather (from *Bacillus subtilis*, pH 10.0, 37 °C, 10 U mg^{-1}; Amano, Nagoya, Japan), and 1 mg ml^{-1} alkaline protease (from *Bacillus pumilus* SMJ-P, pH 10.0, 37 °C, 1,000 U mg^{-1}), respectively. After incubation for 0.5 or 1 h, the residual activity was measured under standard assay conditions. For the control sample, the recombinant enzyme was incubated under the same conditions in the absence of protease.

Kinetic Parameters and Substrate Specificity of Recombinant Xyn11NX

The K_m, V_{max}, and k_{cat} values for the purified recombinant Xyn11NX were determined by measuring the enzyme activity in McIlvaine buffer (pH 7.0) containing 1–10 mg ml^{-1} oat spelt xylan or birchwood xylan as substrate at 55 °C. The data were plotted according to the Lineweaver–Burk method, and then the kinetic constants (K_m, V_{max}, and k_{cat}) were calculated from Lineweaver–Burk plots of the data.

Substrate specificity of the purified enzyme was investigated in the standard assay system containing equal concentration (1%; *w*/*v*) of the following substrates: oat spelt xylan, birchwood xylan, carboxymethyl cellulose (CMC), barley glucan, laminarin, or lichenan.

Hydrolysis Product Analysis

For enzymatic hydrolysis of xylan, the reaction mixture consisting of 11.6 U purified recombinant enzyme and 3 mg xylan (from oat spelt or from birchwood) in 300 μl McIlvaine buffer (pH 7.0) was incubated at 55 °C for 12 h. After hydrolysis, the enzyme was removed from the reaction system using the Nanosep Centrifugal 3 K Device (Pall, East Hills, NY, USA). The products were analyzed by high-performance anion-exchange chromotography (HPAEC) with a model 2500 system from Dionex (Sunnyvale, CA, USA) [13]. Xylose, xylobiose, xylotriose, xylotetraose, and xylopentaose were used as standards.

Nucleotide Sequence Accession Number

The nucleotide sequence for the *N. xinjiangensis* CCTCC AA001025 xylanase gene *xyn11NX* was deposited in the GenBank database under accession number GQ240235.

Results

Gene Cloning and Sequence Analysis

A gene fragment, 494 bp, was amplified by a touchdown PCR using degenerate primers S11F and S11R [10], and the PCR product was cloned into pGEM-T Easy and sequenced. The nucleotide sequence of this fragment shared 72% identity with the xylanse from *Thermopolyspora flexuosa* (BAD02383), indicating that it is a partial xylanase gene. The 5′ and 3′ flanking regions of the core region were amplified by TAIL-PCR using designed nested insertion primers based on the core region sequence. Two PCR products from the 5′ and 3′ flanking regions, which were 645 and 1,016 bp, respectively, were isolated, sequenced, and assembled with the core region. The resulting DNA sequence was 1,814 bp.

One complete chromosomal gene consisting of 726 bp with 66.7% G + C content was identified within the 1,814-bp PCR amplification sequence. The mature protein is 191 residues with a calculated molecular mass of 22 kDa. In the presence of His-tag and additional amino acid residues of the vector, recombinant Xyn11NX is estimated to be about 26 kDa. SignalP analysis indicated the presence of an N-terminal signal peptide at residues 1–50.

Database searches and alignment of the gene and its deduced amino acid sequence with known xylanases showed that the xylanase gene from *N. xinjiangensis* encoded a family 11 xylanase, which only contained a catalytic domain and no carbohydrate-binding module or thermostabilizing domain. The deduced amino acid sequence of Xyn11NX exhibited a high amino acid sequence identities with the xylanases from *Streptomyces thermoviolaceus* (68%; accession no. BAD02383), *Thermobifida fusca* NTU22 (66%; accession no. AAV64879), and *C. thermophilum* (58%; accession no. 1H1A_A; Fig. 1).

The theoretical structure of Xyn11NX has the classical β-jelly roll fold and an α-helix. Two putative catalytic glutamate residues, E136 and E225, are conserved in all of the four sequences (Figs. 1 and 2). Besides, Xyn11NX has another two glutamate residues (E173 and E208) located near the active site, which are different from other GH 11 members (Fig. 1).

Enzyme Expression, Purification, and Mass Spectrometry Analysis

The gene encoding the mature xylanase was expressed in *E. coli*. Significant xylanase activity, 65.5 U ml^{-1},was detected from shaker flask culture after induction with 1 mM IPTG at 20 °C for 16 h, and no xylanase activity was detected in the supernatant of uninduced culture or induced transformant harboring the empty pET-22b(+), confirming that *xyn11NX* encoded a functional xylanase. After 4.12-fold purification (25.6% recovery) with Ni-NTA affinity chromatography, the specific activity of recombinant Xyn11NX was 2,158 U mg^{-1} toward oat spelt xylan. The purified enzyme migrated as a single band of about 26 kDa on SDS-PAGE (Fig. 3), which was the same as the calculated value of recombinant protein.

To identify whether the single band of approximately 26 kDa was the recombinant protein expressed by *xyn11NX*, the band was excised from the SDS-PAGE, digested with trypsin, and analyzed by LC–ESI–MS/MS for peptide finger printing. The amino acid sequences obtained from the mass peaks were compared with the amino acid sequence of Xyn11NX. The peptides GPLVEYYIIEDYGTYRPTGDYK and GTFWSDGSYYDIYETTR completely corresponded to the amino acid sequence of Xyn11NX, confirming that the purified protein was recombinant Xyn11NX.

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TfxA        --------MNHAPASLKSRRRFRPRLLIGKAFAAALVAVVTMIP-STAAHAAVTSNETGY 51
STX-II      --------MNTLVHPQ--GRAGGLRLLVRAAWALALAALAAMMVGGTARADTITSNQTGT 50
CtXyn11A    ----------------------------------------------------XTLTSSATGT 10
Xyn11NX     MTTAQTLPTRTATRPPLPGRVRRLLHRRAVAVAAALAMLCGAVLATPASAQPVYEDTQGY 60
                                                                .: ..  *

TfxA        HDGYFYSFWTDAPGTVSMELGPGGNYSTSWRNTGNFVAGKGWATG-GRRTVTYSASFNPS 110
STX-II      HNGYFYSFWTDAPGTVTMNTGAGGNYSTQWSNTGNFVAGKGWATG-GRRTVTYSGTFNPS 109
CtXyn11A    HNGYYYSFWTDGQGNIRFNLESGGQYSVTWSGNGNWVGGKGWNPGTDNRVINYTADYRPN 70
Xyn11NX     HDGYFYTFWTDAPGTITMNLEPGGSYSTQWGDTGNFVVGKGWSTG-TSRTVDYSATFNPS 119
            *:**:*:****. *.: ::  .**.**. * ..**:* **** .*   *.: *:. :.*.

TfxA        GNAYLTLYGWTRNPLVEYYIVESWGTYRPTG--TYMGTVTTDGGTYDIYKTTRYNAPSIE 168
STX-II      GNAYLALYGWSQNPLVEYYIVDNWGTYRPTG--TYKGTVDSDGGTYDIYMTTRYNAPSIE 167
CtXyn11A    GNSYLAVYGWTRNPLIEYYVVESFGTYDPSTGATRMGSVTTDGGTYNIYRTQRVNAPSIE 130
Xyn11NX     GNGYLALYGWTRGPLVEYYIIEDYGTYRPTG--DYKGTFWSDGSYYDIYETTRVEEPSID 177
            **.**::***::.**:***:::.:*** *:      *:. :**. *:** * * : ***:
                        ▲                                   ■

TfxA        GTRTFDQYWSVRQSKRTSGTITAGNHFDAWARHGMHLGTHDYMIMATEGYQSSGSSNVTL 228
STX-II      GTKTFNQYWSVRQNKRTGGTITTGNHFDAWAAHGMPLGTFNYMILATEGYQSSGSSNITV 227
CtXyn11A    GTKTFYQYWSVRTSKRTGGTVTMANHFNAWRQAGLQLGSHDYQIVATEGYYSSGSATVNV 190
Xyn11NX     GTQTFQQYWSVRHDTRTSGSITTANHFHAWEQAGMPLGTHDYQVMATEGYQSSGSSSVTV 237
            **:** ****** ..**.*::* .***.**   *: **:.:* ::***** ****:.:.:
                                  ■              ▲

TfxA        GTSGGDNPGGGNPPGGGNPPGGGGCTATLSAGQQWNDRYNLNVNVSGSNNWTVTVNVPWP 288
STX-II      GDSGGDNGGGGGGGGGGGNTGG--CTATLSAGEQWSDRYNLNVSVSGSDNWTVTMRVPAP 285
CtXyn11A    G------------------------------------------------------------ 191
Xyn11NX     HTAP---------------------------------------------------------- 241

TfxA        ARIIATWNIHASYPDSQTLVARPNGNGNNWGMTIMHNGNWTWPTVSCSAN 338
STX-II      EKVMATWNVTASYPDAQTLVARPNGNGNNWGVTIQKNGSTTWPTVSCSVG 335
CtXyn11A    ----------------------------------------------
Xyn11NX     ----------------------------------------------
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Fig. 1 Amino acid sequence alignment of the catalytic domains of Xyn11NX (this study) and other family 11 xylanases including a xylanase of *Thermomonospora fusca* (AAV64879), STX-II of *S. thermoviolaceus* OPC-520 (BAD02383), and *C. thermophilum* (1H1A_A) using the ClustalW program. Numbering of the amino acids starts at the N terminus of the proteins. Conserved and identical amino acids are indicated by *asterisks* and *dots*, respectively. The catalytic glutamate residues (E136 and E225) are indicated by *triangles*, and the other two glutamate residues (E173 and E208) near the active center are indicated by *rectangles*. Gaps are indicated by *dashes*

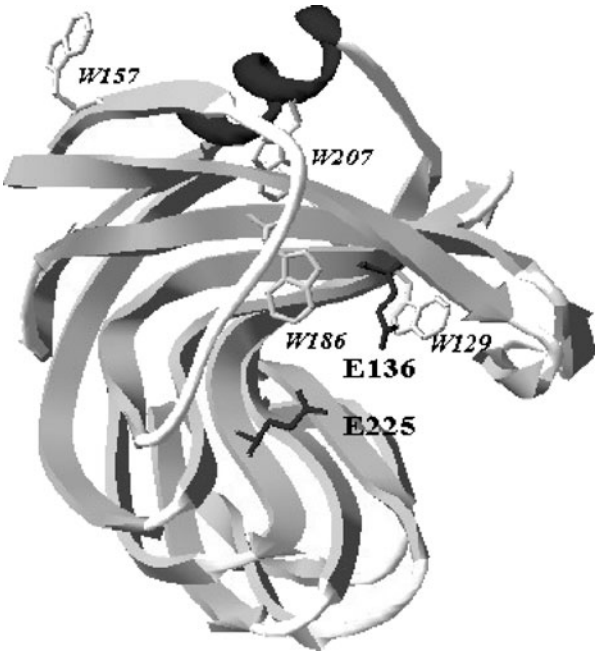

Fig. 2 The predicted tertiary structure of *N. xinjiangensis* Xyn11NX. The predicted catalytic glutamate residues (E136 and E225) and the four tryptophan residues (W129, W157, W186, and W207) near the active site of Xyn11NX resulting from the model have been labeled

Effect of pH and Temperature on Xyn11NX Activity and Stability

The purified recombinant Xyn11NX exhibited optimal activity around pH 7.0 at 55 °C and remained over 60% of the peak activity at pH 6.0–8.0 (Fig. 4a). The enzyme was stable over a wide pH range, retaining more than 80% of the initial activity after incubation in buffers of pH ranging from 5.0 to 11.0 at 37 °C for 1 h (Fig. 4b).

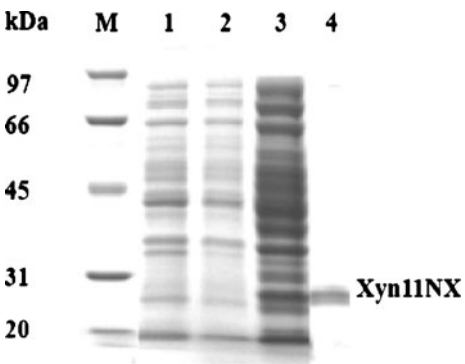

Fig. 3 SDS-PAGE analysis of Xyn11NX expressed in *E. coli* BL21 (DE3). *Lanes M* low molecular weight markers, *1* culture supernatant of *E. coli* pET-22b(+) harboring the empty plasmid after IPTG induction, *2* culture supernatant of pET-*xyn11NX* without IPTG induction, *3* culture supernatant of pET-*xyn11NX* following IPTG induction, *4* purified Xyn11NX after filtration through Ni-NTA chelating column

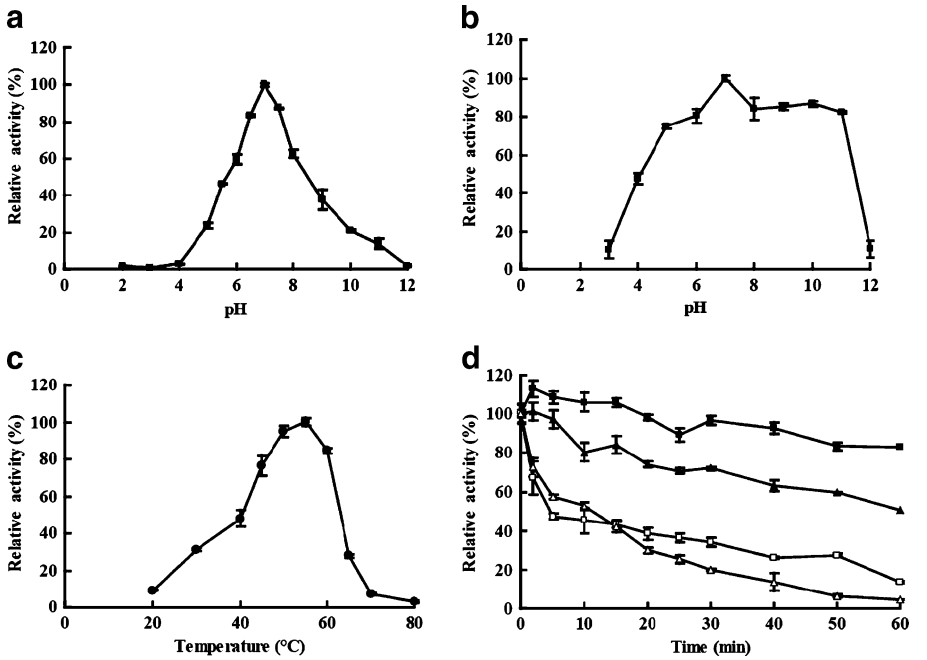

Fig. 4 Characterization of the purified recombinant Xyn11NX. **a** Effect of pH on xylanase activity. The assay was performed at 55 °C in buffers with pH ranging from 2.0 to 12.0. **b** pH stability of xylanase activity. After incubating the enzyme at 37 °C for 1 h in buffers of pH 3.0 to 12.0, the activity was measured in McIlvaine buffer (pH 7.0) at 55 °C. **c** Effect of temperature on xylanase activity measured in McIlvaine buffer (pH 7.0). **d** Thermostability assay. The enzyme was pre-incubated at 60 °C (*filled squares*), 70 °C (*filled triangles*), 80 °C (*empty squares*), or 90 °C (*empty triangles*) in McIlvaine buffer (pH 7.0), and aliquots were removed at specific time points for the measurement of residual activity at 55 °C

The optimal temperature for enzyme activity was 55 °C at pH 7.0, and the enzyme retained more than 80% of the maximum activity when assayed at 45 °C to 60 °C (Fig. 4c). The purified recombinant Xyn11NX was thermostable, retaining more than 80% of the initial activity after incubation at 60 °C for 1 h and more than 40% of the initial activity after incubation at 90 °C for 15 min (Fig. 4d). In the absence of substrate, the half-life of the recombinant Xyn11NX was about 7 h at 60 °C, and at 70 °C its half-life was about 1.5 h.

Effect of Various Chemicals on Xyn11NX Activity

The xylanase activity of the recombinant Xyn11NX in the presence of different metal ions or chemical reagents is shown in Table 2. Cr^{3+} and β-mercaptoethanol at 10 mM enhanced the activity about 1.3- and 1.9-folds, respectively. Na^{+}, K^{+}, Ca^{2+}, Li^{+}, Mg^{2+}, Fe^{2+}, and Mn^{2+} have been found to have a stimulatory effect on xylanase activity. Partial inhibition was observed in the presence of some metal ions at 10 mM concentration, and the enzyme inhibition was in the order of Co^{2+}>Cu^{2+}> Pb^{2+}. The activity was completely inhibited by the presence of Ag^{+} and strongly inhibited by Hg^{2+} even at 1 mM concentration. Addition of other reagents had little or no effect on the activity.

Table 2 Effect of metal ions and chemical reagents on the xylanase activity of purified recombinant Xyn11NX.

Chemicals	Relative activity (%)[a]		
	1 mM	5 mM	10 mM
None	100.0±0.76	100.0±0.15	100.0±0.22
Na^{+}	135.73±1.14	109.37±0.28	110.84±0.75
K^{+}	140.32±0.72	108.03±0.74	137.09±0.65
Ca^{2+}	134.05±0.97	121.8±2.69	112.09±0.46
Li^{+}	138.95±0.65	106.44±0.41	113.66±0.46
Co^{2+}	136.74±0.41	77.3±1.48	47.35±2.72
Cr^{3+}	140.83±0.40	121.32±0.58	130.81±0.57
Ni^{2+}	105.97±0.32	89.31±0.87	84.58±2.46
Cu^{2+}	97.17±0.73	56.02±0.31	51.86±0.12
Mg^{2+}	140.73±0.57	109.15±1.05	122.33±0.43
Fe^{2+}	140.44±1.20	107.16±1.13	116.3±0.80
Mn^{2+}	125.56±0.93	101.51±1.26	42.03±2.45
Zn^{2+}	86.58±0.15	100.18±2.02	100.98±1.75
Pb^{2+}	88.91±0.14	81.20±0.38	57.94±0.45
Ag^{+}	39.41±0.58	38.77±1.63	31.92±1.37
Hg^{2+}	4.76±0.01	2.63±0.12	1.26±0.37
SDS	65.64±0.06	89.03±1.72	97.1±2.01
EDTA	104.61±1.99	95.78±0.26	89.7±0.28
β-Mercaptoethanol	111.21±0.67	135.3±0.30	194.6±0.35

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

Protease Resistance

We also investigated the resistance of recombinant Xyn11NX to neutral and alkaline proteases (Fig. 5). After treatment with subtilisin A at 37 °C for 0.5 h, the enzyme retained more than 80% activity. Incubation with other tested proteases under the same conditions resulted in significant loss of activity—less than 50%. When the incubation time was increased to 1 h, more than 40% initial activity was retained.

Substrate Specificity and Kinetic Parameters of Xylanase

The enzyme had relatively narrow substrate specificity, exhibiting 100% relative activity for oat spelt xylan, 95% for birchwood xylan, and 16% for lichenan, respectively. Almost no activity was detected when the substrate was CMC, barley glucan, or laminarin.

The K_m, V_{max}, and k_{cat} values for oat spelt xylan were 16.08 mg ml^{-1}, 45.66 μmol min^{-1} mg^{-1}, and 18.12 s^{-1}, respectively. When using birchwood xylan as the substrate, the corresponding values were 9.22 mg ml^{-1}, 16.05 μmol $min^{-1}mg^{-1}$, and 6.37 s^{-1}, respectively.

Analysis of Hydrolysis Products

The hydrolysis products of oat spelt xylan and birchwood xylan by recombinant Xyn11NX were analyzed by HPAEC. Xylobiose and xylotriose were the predominant products. The

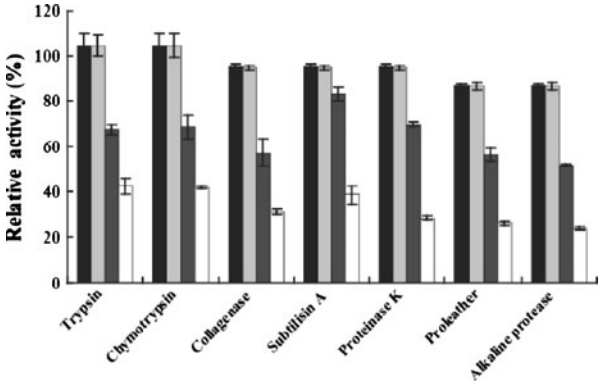

Fig. 5 Effect of proteases on the stability of purified recombinant Xyn11NX. The purified Xyn11NX was incubated with proteases under specific conditions for 30 min (*dark gray*) or 1 h (*white*), and the residual activity was measured in McIlvaine buffer (pH7.0) at 55°C. The purified Xyn11NX was also incubated under the same conditions for 30 min (*black*) or 1 h (*light gray*) without addition of proteases, and the residual activity was measured as controls. The initial xylanase activity represented 100% activity

composition of the hydrolysis products of oat spelt xylan was 2.282 μmol ml^{-1} xylobiose and 0.652 μmol ml^{-1} xylotriose. The product of birchwood xylan was composed of 4.953 μmol ml^{-1} xylobiose and 1.569 μmol ml^{-1} xylotriose.

Discussion

Members of Actinomycetes have been reported to produce a great variety of extracellular enzymes, of which xylanases are of significant industrial importance. These included the xylanases from *Streptomyces* sp. S9 [10], *Micrococcus* sp. AR-135 [14], *S. thermoviolaceus* OPC-520 [15], *Streptomyces halstedii* JM8 [16], *Streptomyces fradiae* var. k11 [17], *Nonomuraea flexuosa* [18], *Streptomyces lividans* 66 [19], and so on. In this study, we first described cloning and expression of a family 11 xylanase, Xyn11NX, from the genus *Nesterenkonia*. This xylanase had high sequence homology to the xylanases from Actinomycetes, but showed much broader pH adaptability and higher thermostability. For example, STX-II from *S. thermoviolaceus* OPC-520 and the xylanase B from *S. lividans* 66 had similar pH optima at 7.0 and 6.5, respectively, but showed almost no activity when assayed at pH below 4.0 or above 9.0 [15, 19]. Some xylanases have similar optimal temperature at 55 °C as Xyn11NX did, such as Xys1L from *S. halstedii* JM8 [16] and SfXyn10 from *S. fradiae* var. k11 [17]. However, Xyn11NX was more thermostable, retaining more than 80% of the initial activity after incubation at 60 °C for 1 h. Under the same conditions, Xys1L and SfXyn10 were almost inactive [16, 17].

Thermostable xylanases have been described in a wide range of microorganisms, such as those from thermophilic *Geobacillus* sp. [20], *Bacillus halodurans* S7 [21], and *Laetiporus sulphureus* [22]. Although the optimum temperature (55 °C) of Xyn11NX was lower than these xylanase, it retained more activity when incubating at elevated temperatures. For instance, the xylanase from thermophilic *Geobacillus* sp. had temperature optimum at 70 °C and retained only 5% activity after pre-incubation at 70 °C for 10 min [20]. The optimum temperature of the xylanase from *B. halodurans* S7 was 75 °C; however, the enzyme had no detectable activity after incubation at 65 °C, pH 10 for 1 h [21]. Grüninger and Fiechter [23] reported a thermostable xylanase with the highest activity at 80 °C, but the half-life of the

enzyme at 80 °C was less than 10 min, and the enzyme retained only 20% activity after pre-incubation without substrate at 80 °C for 2 min, while Xyn11NX retained 40% activity after pre-incubation without substrate at 80 °C for 30 min. As far as GH 11 xylanases are concerned, their thermostability is relatively worse than that of GH 10 xylanases, but recombinant Xyn11NX is good with high thermostability. Thus, the thermal stability of Xyn11NX is an attractive feature for potential industrial applications.

It has been known that xylanase secretion and its extracellular performance depend directly on the type and concentration of ions present in the solution [24]. The enzyme activity was strongly inhibited by Hg^{2+}, however, as reported in other studies [16, 20, 25, 26]. Because Hg^{2+} can oxidize the indole ring, it probably interacts with the tryptophan residues of the enzyme [27]. Tertiary structure prediction indicates that seven tryptophan residues are located in the catalytic domain, and four of them (W129, W157, W186, and W207) are located near the active site of Xyn11NX (Fig. 2). The stimulatory effects of some agents such as Na^{+}, K^{+}, Ca^{2+}, and Li^{+} on enzyme activity might be due to the alteration of structural changes. The enzyme activity remains unaltered in the presence of EDTA, suggesting the possible absence of metal ions at the active site. In addition, the effects of Cu^{2+} and Fe^{2+} are adverse for xylanase application in industries [27]. However, in the presence of 10 mM Cu^{2+} or Fe^{2+}, the enzyme still retained more than 50% activity. These characteristics collectively may facilitate its widespread application in industries. Cr^{3+} stimulated Xyn11NX activity by 31% at 10 mM concentration. The same phenomenon has been reported by Li et al. [17]. The reason might be that Cr^{3+} may change the active site conformation and result in more opportunities of substrate binding on the structure of the active site.

Like the xylanases from *Geobacillus thermoleovorans* and *Thermomyces lanuginosus* [28], Xyn11NX showed no cellulase activity. As cellulase activity may result in poor fiber mechanical strength, xylanases for pulp treatment should be free of cellulolytic activity [29]. Several cellulase-free xylanases have been reported, such as those from *Bacillus amyloliquefaciens* [30] and *B. licheniformis* A99 [31], and most of these xylanases are alkali-liable. On the other hand, many xylanases are known to have binding domain to cellulose or xylan [32–35] and may result in local bleaching by binding to the surface of pulp fibers or re-precipitating xylan. Moreover, binding of such enzymes to cellulose fibers may lead to cellulose disruption [29]. Therefore the cellulase-free Xyn11NX would penetrate easily into the cellulose fiber and allow production of high-quality pulp.

In summary, the favorable properties of Xyn11NX, such as good pH adaptability and thermal stability, cellulase-free natures, and less complex hydrolysis products, make it promising for various applications in many industries, especially in pulp and paper industry.

Acknowledgments This work was supported by the National High Technology Research and Development Program of China (863 Program; No. 2007AA100601), National Key Technology R&D Program of China (No. 2006BAD12B05-03), and Chinese Program on Research for Public Good (Grant No. 2005DIB4J038).

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