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A Xylanase Gene Directly Cloned from the Genomic DNA of Alkaline Wastewater Sludge Showing Application Potential in the Paper Industry

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Abstract A xylanase gene, *aws-2x*, was directly cloned from the genomic DNA of the alkaline wastewater sludge using degenerated PCR and modified TAIL-PCR. The deduced amino acid sequence of AWS-2x shared the highest identity (60%) with the xylanase from *Chryseobacterium gleum* belonging to the glycosyl hydrolase GH family 10. Recombinant AWS-2x was expressed in *Escherichia coli* BL21 (DE3) and purified to electrophoretic homogeneity. The enzyme showed maximal activity at pH 7.5 and 55 °C, maintained more than 50% of maximal activity when assayed at pH 9.0, and was stable over a wide pH range from 4.0 to 11.0. The specific activity of AWS-2x towards hardwood xylan (beechwood and birchwood xylan) was significantly higher than that to cereal xylan (oat spelt xylan and wheat arabinoxylan). These properties make AWS-2x a potential candidate for application in the pulp and paper industry.

Keywords Xylanase · Direct cloning and heteroexpression · Alkaline wastewater sludge · Hardwood xylan

Introduction

Xylan is the major hemicellulose of plant cell walls and is composed of a backbone of β-1,4-linked xylose residues decorated with arabinofuranose, acetyl, 4-*O*-methyl D-glucuronic acid

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or ferulate [1]. Xylan exists in plants in various forms, *O*-acetyl-4-*O*-methylglucuronoxylan in hardwood, arabino-4-*O*-methylglucuronoxylan in softwood, and arabinoxylan in grasses and annual plants [2]. The complete degradation of xylan requires a large variety of cooperatively acting enzymes, in which endo-1,4-β-D-xylanase (EC 3.2.1.8) is the crucial enzyme that randomly cleaves the β-1,4-D-linkages in xylan backbone, β-D-xylosidase cleaves the xylose monomers from the non-reducing end of xylo-oligosaccharides and xylobiose, and α-L-arabinofuranosidase, α-D-glucuronidase, acetylxylan esterase, ferulic acid esterase, and *p*-coumaric acid esterase catalyze the removal of the side groups [3]. Within CAZY classification system (http://www.cazy.org/fam/acc_GH.html), xylanases have been confined into glycoside hydrolase (GH) families 5, 7, 8, 10, 11, and 43, and the majority of the xylanases belongs to either GH 10 or GH 11 [2].

Xylanases have been reported from bacteria, fungi, and plants [4]. Those from microorganisms have attracted a great deal of attention for application in various industrial processes, such as food, feed, environmental science (bio-fueling, effluent treatment, and agro-waste treatment), and pulp and paper industries [5]. In the pulp and paper industry, xylanases showing optimal activity in alkaline conditions are preferred to selectively remove xylan from the surface and pores of fibers for bleaching [6]. However, so far only several alkaline xylanases have been reported, such as the xylanases from *Bacillus* sp. [7]. To obtain more favorable xylanases, culture-independent methods are widely used to enhance the opportunity, and some xylanase genes have been directly cloned from the environment DNA [8]. For instance, xylanase genes from the environmental DNA of a hot pool were retrieved by a modified genomic walking PCR technique [9]. A novel β-1,4-xylanase gene was obtained from the genomic DNA mixture of human fecal bacteria [10]. Because microorganisms may adapt to the extreme environment by producing some adaptive enzymes, the extreme alkaline environment might be a valuable microbial source for alkaline xylanase genes.

In this study, we collected an alkaline wastewater sludge sample (pH 9.0) from a pulp mill, directly cloned a GH 10 xylanase gene (*aws-2x*) from the environmental genomic DNA and expressed the gene in *Escherichia coli*. The recombinant enzyme AWS-2x exhibited maximum activity at pH 7.5 and excellent stability over a broad pH range (pH 4.0–11.0) and showed higher specific activity towards beechwood xylan and birchwood xylan than to oat spelt xylan and wheat arabinoxylan. These properties of AWS-2x are favorable for potential application in the pulp and paper industry.

Materials and Methods

Strains, Vectors, and Medium

E. coli JM109 (TaKaRa, Japan) was used for the construction and propagation of recombinant plasmids. *E. coli* BL21 (DE3) (Novagen, Germany) was used as the host for heterogeneous expression. The pGEM-T Easy vector (Promega, USA) and pET-22b(+) (Novagen) were used for gene cloning and expression, respectively. The Genome Walking kit, Purification kits, LA Taq DNA polymerase with LA buffer, and restriction endonucleases were purchased from TaKaRa. T4 DNA ligase was purchased from New England Biolabs (UK). The substrates oat spelt xylan, beechwood xylan, birchwood xylan, and wheat arabinoxylan were purchased from Sigma (USA). Luria–Bertani (LB) medium containing 100 μg ml^{-1} ampicillin was used to culture *E. coli* strains at 37 °C. All the other chemicals were of analytical grade and commercially available.

Sample Collection and DNA Extraction

The alkaline wastewater sludge sample (1 l) was collected from a paper mill in Shanxi Province, China, and stored at −20 °C before use. The pH value of the sample was determined to be 9.0.

To extract the environmental genomic DNA, 200 ml of the mixture was centrifuged at 12,000×*g* for 10 min, and the sediment was ground into a fine powder using mortar and pestle in the presence of liquid nitrogen. The ground sample was added into 20-ml lysis buffer (0.1 M Tris-HCl, 0.1 M EDTA, 1.5 M NaCl, 2% CTAB, and 1% SDS), digested with 80-μl protease K (200 mg ml^{-1}), and incubated at 70 °C for 3 h with gentle shaking every 30 min. The environmental genomic DNA was precipitated in accordance with the method for DNA extraction from soil samples [11], and purified using the Purification kits. The yield of the purified environmental genomic DNA was about 0.75 μg ml^{-1} sample.

Direct Cloning of the Xylanase Gene *aws-2x*

Using the environment genomic DNA as template, a touchdown PCR using the degenerate primers XF1 (see Table 1) and X_{10}-R [12] was performed to obtain the core region between the conserved blocks [W/Y]-D-W-D-V-[V/C/N]-N-E and [D/H]-[G/A/C]-[I/V/L]-G-[M/F/L/I]-Q-[S/G/M/C]-H (about 81 amino acids between). The PCR product, approximately 240 bp in length, was gel purified, ligated with pGEM-T Easy vector and sequenced by Sangon (China). To obtain the full-length xylanase gene *aws-2x*, a modified thermal asymmetric interlaced (TAIL)-PCR [13] was performed to obtain the 5′ and 3′ flanking regions with the nested insertion-specific primers (usp1-3 and dsp1-3; Table 1), respectively. All PCR products were electrophoresed in 1.2% agarose gels, and those showing expected sizes were sequenced. Sequence assembly was analyzed by Vector NTI Suite 7.0 software. Primers AWS-2X-QUAN-F and AWS-2X-QUAN-R (Table 1) were designed based on the assembled DNA sequence and used to clone the full-length *aws-2x* from the environment genomic DNA directly. Alignments of DNA and protein sequences were conducted with Blastn and Blastp programs, respectively (http://www.ncbi.nlm.nih.gov/BLAST/). The signal peptide was predicted using SignalP (http://www.cbs.dtu.dk/

Table 1 Primers used in this study

Primers	Sequences (5′→3′)[a]
XF1	GATTGGGACGTNGTNAAYGARGT
Xyl2usp-1	GCCTTGCTCCTGCAACGACTTTACCATACGG
Xyl2usp-2	GCCTCACGTTTACCCTTCCTTGCCATCGAAAAATC
Xyl2usp-3	CAATTCTGCATCAGGATCAGCTTCATGTGCAAACTGGAAAG
Xyl2dsp-1	CGGTGAATGGCGTAAAAGCAAGTTTTACCAGATTATTGGTGAG
Xyl2dsp-2	CTGGCTTTCCAGTTTGCACATGAAGCTGATCCTGATG
Xyl2dsp-3	GGCCGTTGTCCGTATGGTAAAGTCGTTGCAG
AWS-2X-QUAN-F	ATGATTATGATTATGAAAAAATGTATTTGCTATTT
AWS-2X-QUAN-R	TTATTTGACTTCAGATATCTTGCCTTCAAC
pET22-2-x-s-SF	GCCGAGCTCCTCGGAGAAACAGGAAAAAGAGCCG
pET22-2-x-s-NR	GCCGCGGCCGCTTTGACTTCAGATATCTTGCCTTCAACTTG

[a] Restriction sites are underlined and R=A/G, N=A/C/G/T, and Y=C/T

services/SignalP/). Homology modeling was performed with Discovery Studio 2.5 MODELER (USA), using xylanase TaXyn10 (PDB code: 2BNJ) from *Trichoderma aurantiacus* as the template, to show the binding site with the arabinose side chain of arabinoxylan.

Expression of *aws-2x* in *E. coli*

The mature protein coding sequence was amplified from the environmental genomic DNA of the alkaline wastewater sludge using primers pET22-2-x-s-SF and pET22-2-x-s-NR (Table 1). The PCR product was gel purified, digested with *Sac*I and *Not*I, and cloned into the corresponding site of vector pET-22b(+). The recombinant plasmid with a C-terminal-fused six His tag, pET-*aws-2x*, was transformed into *E. coli* BL21 (DE3) competent cells. Positive transformants were picked up from single colonies and grown in fresh LB medium containing 100 μg ml^{-1} ampicillin overnight at 37 °C. The positive transformant harboring pET-*aws-2x* was identified by PCR analysis, and confirmed by DNA sequencing. The culture of positive transformant was then inoculated into fresh LB medium (1:100 dilutions) containing ampicillin and grown aerobically at 37 °C to an A_{600} of 0.6–1.0. Isopropyl-β-D-1-thiogalactopyranoside (IPTG) was then added to a final concentration of 0.8 mM for induction. After incubation for an additional 20 h at 18 °C, the cells were collected by centrifugation at 12,000×*g*, 4 °C for 5 min.

Purification of the Recombinant Xylanase

Total protein was extracted from the recombinant *E. coli* cells using Bugbuster Protein Extraction Reagent (Novagen), followed by centrifugation at 13,000×*g*, 4 °C for 10 min. The crude enzyme preparation (5–10 ml) was loaded onto a Ni-NTA chelating column (Qiagen, Germany) that had been equilibrated with buffer A (20 mM Tris-HCl, 500 mM NaCl, and 10% (*w*/*v*) glycerol, pH 7.6). Proteins were eluted using a linear gradient of imidazole (0, 20, 40, 60, 80, 100, 200, and 500 mM) in the same buffer. Fractions with enzyme activity were pooled and analyzed by 12% (*w*/*v*) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 80 V, 3 h) with Coomassie brilliant blue R-250 staining. The protein concentration was determined using the Bradford method [14] and using bovine serum albumin as a standard.

Enzyme Activity Assay

The 3,5-dinitrosalicylic acid (DNS) method was used to determine the xylanase activity [15]. The reaction system contained 0.1 ml of appropriately diluted enzyme and 0.9 ml McIlvaine buffer (0.2 M Na_2HPO_4/0.1 M citric acid; pH 7.5) containing 1% (*w*/v) oat spelt xylan. After incubation at 55 °C for 10 min, the reaction was stopped by addition of 1.5 ml DNS reagent. The mixture was then boiled for 5 min and cooled to room temperature, and the absorption at 540 nm was measured. One unit of xylanase activity was defined as the amount of enzyme that released 1 μmol of reducing sugar equivalent to xylose per minute.

Biochemical Characterization

The optimal pH of the purified recombinant AWS-2x was determined at 55 °C in buffers of pH 3.0 to 12.0. The enzyme stability at different pH levels was estimated by measuring the residual enzyme activity after incubating the enzyme solution in buffers at pH 3.0–12.0,

37 °C for 1 h. The buffers used were McIlvaine buffer (pH 3.0–8.0), 0.1 M Tris-HCl (pH 8.0–9.0), and 0.1 M glycine-NaOH (pH 9.0–12.0).

The optimal temperature for AWS-2x activity was determined over the temperature range of 30–70 °C in McIlvaine buffer at the optimal pH. The thermostability of recombinant AWS-2x was determined by measuring the residual activity under standard conditions (pH 7.5, 55 °C, 10 min) after pre-incubation of the enzyme in McIlvaine buffer (pH 7.5) at 55 or 60 °C without substrate for various periods.

The effect of various metal ions and chemical reagents on the activity of purified recombinant AWS-2x was determined under standard conditions in the presence of 5 or 10 mM of NaCl, KCl, $CaCl_2$, LiCl, $CoCl_2$, $CrCl_3$, $NiSO_4$, $CuSO_4$, $MgSO_4$, $FeCl_3$, $MnSO_4$, $ZnCl_2$, $PbCl_2$, $AgNO_3$, $HgCl_2$, SDS, EDTA, or β-mercaptoethanol. The system without any chemicals was treated as blank control.

Each experiment included three replicate samples.

Substrate Specificity and Kinetic Parameters

The substrate specificity of purified recombinant AWS-2x was determined using 10 mg ml^{-1} oat spelt xylan, beechwood xylan, birchwood xylan, and wheat arabinoxylan as substrates.

Kinetic parameters for the purified recombinant AWS-2x were determined under standard conditions (pH 7.5, 55 °C) for 5 min using 1–10 mg ml^{-1} beechwood xylan as the substrate. The apparent K_m and V_{max} were determined from a Lineweaver–Burk plot using the non-linear regression computer program GraphPad Prism. Three independent experiments were averaged, and each experiment included three replicate samples.

Analysis of Hydrolysis Product

The reaction mixture containing 3 U of purified recombinant enzyme and 5 mg ml^{-1} oat spelt xylan or beechwood xylan in 500 μl McIlvaine buffer (pH 7.5) was incubated at 37 °C for 24 h. After hydrolysis, the enzyme was removed from the reaction system using the Nanosep Centrifugal 3 K Device (Pall, USA). The hydrolysis products were analyzed by high-performance anion-exchange chromatography (HPAEC) with a Dionex model 2500 system (USA). Xylose, xylobiose, xylotriose, xylotetraose, and xylopentaose were used as standards [16].

Accession Number

The xylanase gene, *aws-2x*, directly cloned from the genomic DNA of the alkaline wastewater sludge was deposited in the GenBank under accession number of HQ207192.

Results

Gene Cloning and Sequence Analysis of *aws-2x*

A total of 23 gene fragments of about 250 bp in length were amplified directly from the genomic DNA of alkaline wastewater sludge using the degenerate primers XF1 and X_{10}-R. Among them, a 243-bp gene fragment showing 71% identity to a family 10 xylanase from uncultured organism (GenBank accession no. ACL37205.1) was selected for full-length gene cloning. The 5′ and 3′ flanking regions of this fragment were successfully obtained by

TAIL-PCR, and assembled with the corn region to give a 1,562 bp DNA sequence. A 1,224-bp opening reading frame of *aws-2x* was identified, and further confirmed by primers AWS-2X-QUAN-F and AWS-2X-QUAN-R (Fig. 1). *aws-2x* encoded a 407-residue polypeptide including a putative signal peptide at residues 1–25. The mature protein consisted of 382 residues with a calculated molecular mass of 43.9 kDa and an estimated isoelectric point of 5.75.

aws-2x encoded a single-domain enzyme belonging to GH 10. The deduced amino acid sequence of AWS-2x had highest identity of 60% with an endo-1,4-β-xylanase from *Chryseobacterium gleum* ATCC35910 (GenBank accession no. ZP_03852841.1) and 56% with a xylanase from the symbiotic *Sphingobacterium* sp. TN19 (GenBank accession no. ACX30652.1) (Fig. 2). Homology modeling using Discovery Studio identified two residues, Asn25 and Arg26, close to the arabinose side chain.

Expression and Purification of AWS-2x in *E. coli*

Recombinant AWS-2x was expressed in *E. coli* BL21 (DE3) competent cells. After induction with IPTG at 18 °C for 20 h, significant xylanase activity, 0.18 Uml^{-1}, was detected in the culture supernatant of recombinant *E. coli* harboring pET-*aws-2x*. No xylanase activity was detected in the medium of the uninduced culture or induced transformant harboring the empty plasmid pET-22b(+). The cell lysate obtained with Bugbuster Protein Extraction Reagent showed xylanase activity of 7.37 Uml^{-1}.

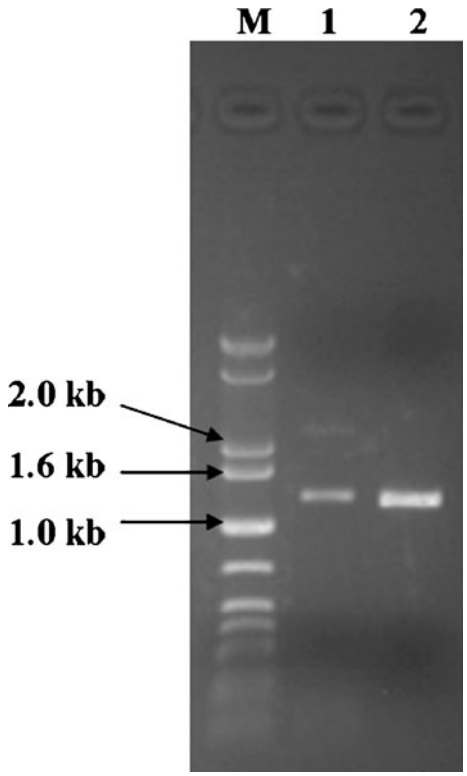

Fig. 1 Polyacrylamide gel electrophoresis analysis of the full-length *aws-2x* and its PCR products for recombinant expression. *Lanes M*, molecular mass standard; *1*, PCR products of full-length *aws-2x*; *2*, PCR products of mature AWS-2x-coding sequence for recombinant expression

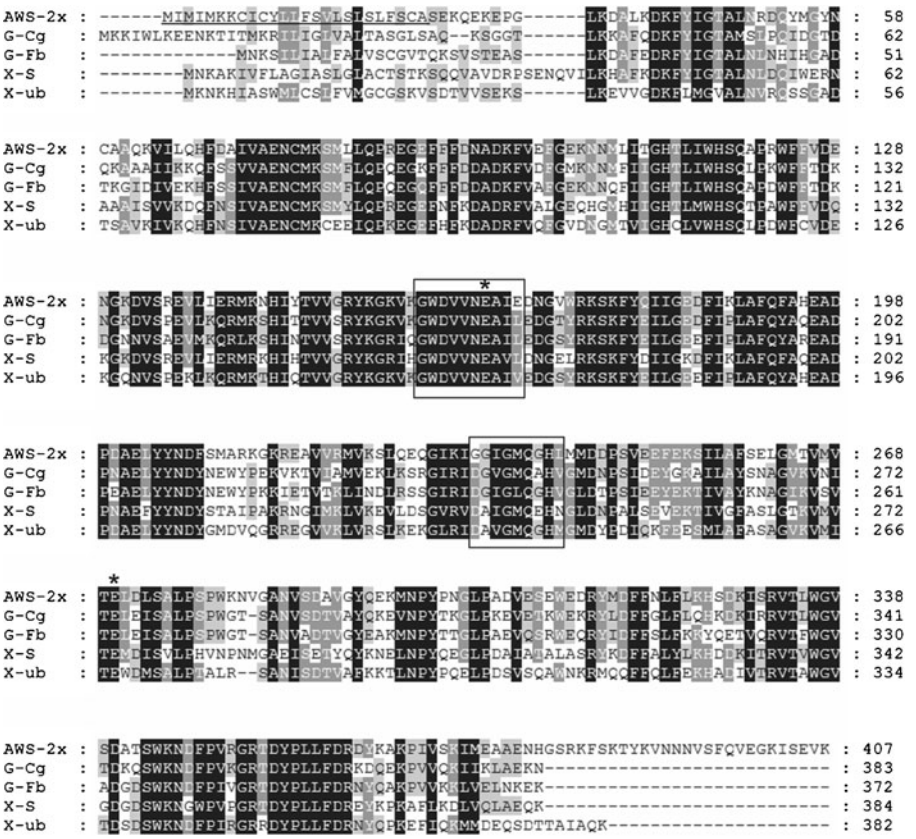

Fig. 2 Amino acid sequence alignment of AWS-2x from the alkaline wastewater sludge with the endo-1,4-β-xylanase from *Chryseobacterium gleum* ATCC 35910 (ZP_03852841.1) (*G-Cg*), endo-1,4-β-xylanase A from *Flavobacteriaceae bacterium* 3519-10 (YP_003096238.1) (*G-Fb*), endo-1,4-β-xylanase from *Sphingobacterium* sp. TN19 (ACX30652.1) (*X-S*) and β-1,4-xylanase from an uncultured bacterium (BAD80892.1) (*X-ub*). The signal peptide is underlined. Identical and similar amino acids are indicated by black and gray shading, respectively. The predicted catalytic glutamate residues are indicated by asterisks. The conserved sequences of GH 10 xylanases are boxed

Recombinant AWS-2x was purified to electrophoretic homogeneity by Ni^{2+}–NTA metal chelating affinity chromatography (Fig. 3). The purified enzyme migrated a single band on SDS-PAGE with a molecular mass of about 45.0 kDa, which was identical to the calculated value plus the weight of His tag. The specific activity of the purified recombinant AWS-2x was 84.93 Umg^{-1} towards beechwood xylan.

Biochemical Characterization

Purified recombinant AWS-2x exhibited optimal activity at pH 7.5, and more than 50% of the maximum activity was retained at pH 6.5–9.0 (Fig. 4a). The optimal temperature for the enzyme activity was 55 °C at pH 7.5 (Fig. 4b). The enzyme was stable over a broad pH range, retaining more than 60% of the initial activity after incubation in buffers ranging from pH 4.0 to 11.0 at 37 °C for 1 h (Fig. 4c). After incubation at 55 °C for 1 h, the enzyme

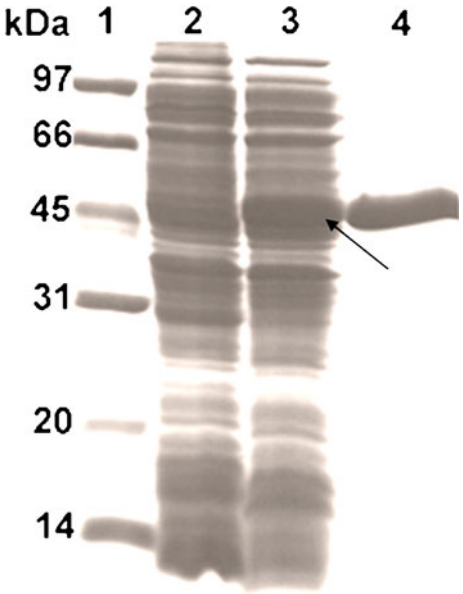

Fig. 3 SDS-PAGE analysis of expression and purification of recombinant AWS-2x. *Lanes 1*, molecular mass standard; *2*, culture supernatant of the induced transformant harboring the empty plasmid pET-22b(+); *3*, culture supernatant of the induced transformant harboring pET-*aws-2x* (the target protein marked with an arrow); *4*, purified AWS-2x using Ni^{2+}-NTA metal chelating affinity chromatography

retained more than 90% of the initial activity. When treated at 60 °C for 30 min, the enzyme only retained about 13% activity (Fig. 4d).

The effect of different metal ions and chemical reagents on the enzymatic activity of purified recombinant AWS-2x was shown in Table 2. The activity was absolutely inhibited by SDS and Hg^{2+}, and partially inhibited by Cu^{2+} and Ag^{+}. Mn^{2+} and β-mercaptoethanol enhanced the activity about 1.2- and 1.7-fold at 5 mM, respectively.

Substrate Specificity and Kinetic Parameters

The specific activity of recombinant AWS-2x towards various substrates was shown in Table 3. The highest activity was observed with beechwood xylan, followed by birchwood xylan, wheat arabinoxylan, and oat spelt xylan. No activity towards CMC was detected. The kinetic parameters, K_m and V_{max}, of purified recombinant AWS-2x with respect to beechwood xylan were determined to be 12.67 mg ml^{-1} and 432.28 μmol^{-1} min^{-1} mg^{-1}, respectively.

Analysis of Hydrolysis Product

HPAEC analysis of the hydrolysis products of AWS-2x showed that xylobiose and xylose were the main products of beechwood xylan and oat spelt xylan. The composition of the hydrolysis products of beechwood xylan was 21.95% xylose, 67.40% xylobiose and 10.65% xylan polymer. The hydrolysis products of oat spelt xylan were comprised of 20.78% xylose, 59.74% xylobiose and 19.48% xylan polymer. This result confirmed that AWS-2x was an endo-xylanase.

Discussion

Microbial extracellular enzymes always have some adaptive properties to the habitats that microorganisms are colonized [2]. For instance, xylanase XYL10C from acidophilic

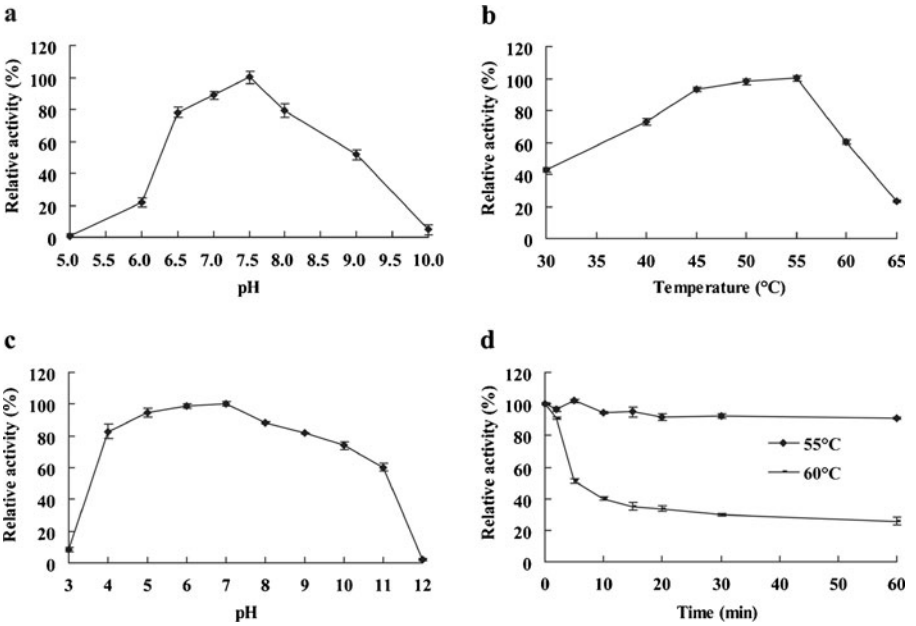

Fig. 4 Characterization of the purified recombinant AWS-2x. **a** Effect of pH on AWS-2x. The assay was performed at 55 °C in buffers ranging from pH 5.0 to 10.0. **b** Effect of temperature on AWS-2x measured in McIlvaine buffer (pH 7.5). **c** pH stability of AWS-2x. After pre-incubating the enzyme at 37 °C for 1 h in buffers of pH 3.0–12.0, the activity was measured in McIlvaine buffer (pH 7.5) at 55 °C. **d** Thermostability of recombinant AWS-2x. The enzyme was pre-incubated at 55 and 60 °C in McIlvaine buffer (pH 7.5) without substrate, and aliquots were removed at specific time points for the measurement of residual activity at 55 °C

Bispora sp. MEY-1 that was isolated from the acidic wastewater of a uranium mine had high activity and good stability under acidic conditions (pH 1.5–6.0) [17]. The alkaliphilic *Bacillus* sp. AR-009 isolated from an alkaline soda lake produced an alkaline xylanase [18]. In this study, we selected the alkaline wastewater sludge of a paper mill as the source of alkaline xylanase genes. Wastewater of paper mill has been reported to be a good source for alkaliphiles, from which alkaliphilic *Bacillus firmus* [19] and a *Proteobacteria* strain CKB [20] have been isolated. Because only a small fraction (<1%) of the naturally occurring microorganisms can be cultivated using standard techniques [21], it's more efficient to obtain objective genes by construction of environmental DNA libraries and direct screening of functional enzyme-encoding genes [22]. In this study, we isolated the genomic DNA from the alkaline wastewater sludge, and directly cloned xylanase gene fragments by using degenerate PCR and TAIL-PCR techniques. As a result, 23 xylanse gene fragments and seven full-length xylanase genes were obtained, four full-length genes including AWS-2x were expressed in *E. coli*, and all the recombinant enzymes showed good stability under alkaline conditions (data not shown). Our results indicate that direct cloning of objective genes from the environmental DNA greatly enhances the opportunities to take full advantage of the enormous naturally occurring microbial resources [8].

In general, endo-xylanases from fungi exhibit peak activity at pH 4.0–6.5 [23], and those from bacteria are highly active at pH 5.0–8.0 [4]. Recombinant AWS-2x exhibited the highest activity at pH 7.5, suggesting its probable microbial source of bacteria. The high sequence identity of AWS-2x with bacterial xylanases from *C. gleum* ATCC35910 and symbiotic *Streptomyces* sp. TN19 further confirmed its bacterial source. Some strains of

Table 2 Effect of metal ions and chemical reagents on the activity of purified recombinant AWS-2x

Chemicals	Relative activity (%)[a]	
	5 mM	10 mM
None	100	100
Mn^{2+}	120.44±2.98	125.64±2.73
Co^{2+}	113.69±2.64	95.93±1.54
Cr^{3+}	104.41±2.23	97.42±3.23
Fe^{3+}	104.10±4.57	119.65±2.11
K^{+}	103.77±1.54	114.76±0.41
Zn^{2+}	100.88±2.41	109.38±1.01
Ni^{+}	100.04±1.78	95.72±2.31
Mg^{2+}	99.78±0.85	106.20±1.46
Na^{+}	99.47±3.32	106.46±1.15
Ca^{2+}	97.00±3.62	95.62±2.00
Li^{+}	92.34±1.58	101.09±0.29
Pb^{2+}	91.14±3.55	92.16±1.04
Ag^{+}	64.17±8.50	61.80±1.46
Cu^{2+}	51.06±1.85	31.69±1.49
Hg^{2+}	0	0
β-Mercaptoethanol	172.60±4.95	134.39±3.30
EDTA	102.60±1.82	96.18±3.75
SDS	0	0

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

Chryseobacterium defluvii have been isolated from the wastewater or activated sludge [24], but no studies are conducted to characterize *Chryseobacterium* xylanases. Compared with XynA19 from *Streptomyces* sp. TN19 that exhibited >60% of the maximum activity at pH 5.5–7.5 and retained more than 55% of the initial activity after incubation at pH 5.0 to 9.0 for 1 h [25], recombinant AWS-2x showed higher activity (>80%) at a broader pH range (pH 6.5–8.0), and retained more than 80% of the initial activity under the same conditions. Although without typical alkalophilic properties, AWS-2x exhibited high relatively activity under alkaline conditions (50% activity at pH 6.5–9.0), and was stable over a broad pH range (pH 4.0–11.0). These properties implied its potential applications in the paper industry.

Table 3 Substrate specificity of purified recombinant AWS-2x

Substrate	Specific activity (U mg^{-1})[a]	Relative activity (%)
Oat spelt xylan	36.10±2.18	100
Wheat arabinoxylan	61.88±2.20	171.3
Birchwood xylan	70.59±3.57	195.5
Beechwood xylan	84.93±3.17	235.3
Carboxymethyl cellulose (CMC)	–	–

[a] Values represent the means±SD (n=3)

The hydrolysis capacity of xylanase is variable towards different substrates. For example, the xylanase from *Plectosphaerella cucumerina* was more active on oat spelt xylan than on birchwood or beechwood xylan [26]. The purified xylanase from *Bacillus* sp. showed higher specific activities on birchwood xylan and beechwood xylan than on oat spelt xylan [27]. In this study, AWS-2x showed significantly higher ability to catalyze the hydrolysis of beechwood xylan and birchwood xylan (235% and 195%, respectively) than of wheat arabinoxylan (171%) and oat spelt xylan (100%) (Table 3). This tremendous difference is probably ascribed to the composition and structural differences of xylans—glucuronoxylan in hardwood and arabinoxylan in wheat and other cereals [28]. Analysis of the hydrolysis products from glucuronoxylan and arabinoxylan shows that family 10 xylanases attack the glycosidic linkage next to the branch and towards the non-reducing end and require two unsubstituted xylose residues between the branches [29]. This selective cutting style indicates that GH 10 xylanases have higher capacity to degrade xylan with less side chain substitution in the backbone. Because glucuronoxylan is less branched and arabinoxylan is more decorated, it might be the reason why AWS-2x possesses higher catalytic activity on hardwood xylan. Moreover, the extent of arabino decorations in wheat arabinoxylan was supposed to be higher than oat spelt xylan. Residues Asn25 and Arg26 in xylanase TaXyn10 from *T. aurantiacus* play key roles in arabinose recognition, but are not conserved in other GH 10 xylanases [1]. Homology modeling with TaXyn10 as the template identified these two residues in AWS-2x, which are located close to the arabinose side chain. However, the recognition and binding mechanisms of xylan and AWS-2x requires further studies.

In this study, we obtained a new family 10 xylanse gene, *aws-2x*, by directly cloning from the genomic DNA of the alkaline environment. Recombinant AWS-2x in *E. coli* exhibited some alkaline properties, such as high activity and excellent stability in alkaline conditions. The enzyme showed higher specific activity towards hardwood xylan than to cereal xylan. These properties make AWS-2x potential for application in the paper industry. Further study of the relationship between structure and function of AWS-2x may reveal the xylan hydrolysis mechanism of this enzyme, and provide better ideas for efficient degradation of hardwood xylan.

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