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Cloning, Expression, and Characterization of a New *Streptomyces* sp. S27 Xylanase for Which Xylobiose is the Main Hydrolysis Product

Ning Li · Pengjun Shi · Peilong Yang · Yaru Wang · Huiying Luo · Yingguo Bai · Zhigang Zhou · Bin Yao

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Abstract A xylanase gene, *xynBS27*, was cloned from *Streptomyces* sp. S27 and consisted of 693 bp encoding a 230-residue protein, including a putative 41-residue signal peptide. Belonging to the glycoside hydrolase family 11, XynBS27 exhibits the maximum identity (75.9%) to the xylanase from *Streptomyces* sp. zxy19. Recombinant XynBS27 was overexpressed in *Pichia pastoris*, and the xylanase activity was 7624.0 U/ml after high-cell-density fermentation in 3.7-L fermenter. The purified recombinant XynBS27 had a high specific activity of 3272.0 U/mg. The optimum temperature and pH for XynBS27 activity was 65 °C and pH 6.5, respectively. XynBS27 showed good pH stability and retained more than 80% of the maximum activity after incubation in buffers with pH ranging between 4.0 and 12.0 at 37 °C for 1 h. The main hydrolysis product of xylan by XynBS27 was xylobiose (>75%), which was good for human health derived from its ability to modulate the intestinal function. The attractive biochemical characteristics of XynBS27 suggest that it may be a good candidate in a variety of industrial applications.

Keywords *Streptomyces* sp. S27 · Xylanase · Overexpression · *Pichia pastoris* · Xylobiose

Introduction

Xylan, the major component of the hemicellulose complex of the plant cell wall, is a heterogeneous polysaccharide with a linear backbone comprised of β-1,4-D-xylopyranoside residues [1]. Among the various xylanolytic enzymes, endo β-1,4-xylanase (EC 3.2.1.8) is crucial for depolymerization of the main backbone of xylan. Xylanase randomly catalyzes the endohydrolysis of β-1,4-D-xylosidic linkages in xylan into short xylo-oligosaccharides [2]. Xylo-oligosaccharides are sugar oligomers made up of xylose units and could be used in food, feed, pharmaceutical, and agricultural fields. For food applications, xylobiose is

N. Li · P. Shi · P. Yang · Y. Wang · H. Luo · Y. Bai · Z. Zhou · B. Yao (✉)
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, People's Republic of China
e-mail: yaobin@caas-bio.net.cn

considered to be a xylo-oligosaccharide and has been used as a food ingredient to modulate the intestinal function since xylo-oligosaccharides could selectively be used by the beneficial gastrointestinal microflora and suppress the growth of pathogenic bacteria [3]. Xylanases have been used for enzymatic production of xylo-oligosaccharides and have various potential applications in food, animal feed, textile, waste treatment, paper, and biofuel industries [4].

Glycoside hydrolase (GH) family 11 xylanases are commonly found in fungi, bacteria, and actinomyces. *Streptomyces* has been recognized as the dominant xylanolytic species of actinomyces that produce enzymes involved in hemicellulose degradation, which have important industrial applications [1]. Xylanases from *Streptomyces* are mainly endo-type, which have been reported in *Streptomyces lividans*, *Streptomyces flavogriseus*, *Streptomyces olivaceoviridis*, *Streptomyces thermonitrificans*, and *Streptomyces cyaneus* [5–9].

In the present study, we isolated a *Streptomyces* sp. strain, designated S27, which exhibited xylanolytic activity. A new GH11 xylanase gene (*xynBS27*) was cloned from *Streptomyces* sp. S27, overexpressed in *Pichia pastoris*, and the recombinant enzyme was characterized.

Materials and Methods

Strains and Media

The strain *Streptomyces* sp. S27 was isolated from the soil of Flaming Mountain in the Turpan Basin of Xinjiang, China and identified by using molecular method (GenBank accession no. EU660497 for 16S rDNA sequence). It was deposited in the Agricultural Culture Collection of China (ACCC) under registration number ACCC41168.

Escherichia coli JM109, which was obtained from Promega (USA), was used for cloning, transformation, and propagation. The host strain *P. pastoris* GS115 was obtained from Invitrogen (USA). The ingredients of regeneration dextrose base plate (RDB), minimal methanol medium (MM), minimal dextrose medium (MD), buffered glycerol-complex medium (BMGY), buffered methanol-complex medium (BMMY), and the culture conditions for *P. pastoris* are provided in the *Pichia* Expression Kit manual (Invitrogen, version M).

Polymerase Chain Reaction Amplification, Cloning, and Sequencing of the Xylanase Gene

Based on the alignment of the amino acid sequences of GH11 xylanases from *Streptomyces* (http://www.cazy.org/fam/GH11.html), two highly conserved regions (YSFWTD and EGYQSS) flanking a fragment of approximately 170 amino acids residues were identified. Two degenerate primers (S11F, 5′-TACTCSTTCTGGACSGAC-3′; S11R, 5′-CTGCTCTGRTANCCYTC-3′) were designed to amplify this region from the genomic DNA of *Streptomyces* sp. S27. Polymerase chain reaction (PCR) was performed as follows: 94 °C, 5 min; 30 cycles of 94 °C, 30 s, 50 °C, 30 s, and 72 °C, 30 s; with a final extension at 72 °C for 7 min. The amplified fragment (about 500 bp) was purified and ligated into the pGEM-T Easy vector for sequencing and BLAST analysis.

A genomic library was constructed as described [10]. A colony PCR method was used to isolate the target gene with two specific synthetic primers: SF (5′-TGGTGGAGTACTAC ATCGTCGACAAC-3′) and SR (5′-GGTCGCCATGATCATGTAGTAGTTG-3′). A positive clone was obtained, and the recombinant plasmid was isolated and sequenced. The

nucleotide sequence for the xylanase gene from *Streptomyces* sp. S27 was deposited in GenBank under the accession no. EU660500.

Sequence Analysis

The sequence assembly was performed using the Vector NTI Suite 7.0 software, and the nucleotide sequence was analyzed using the National Center for Biotechnology Information open reading frame (ORF) Finder tool (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The signal peptide in the deduced amino acid sequence was predicted by the 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, respectively (http://www.ncbi.nlm.nih.gov/BLAST/). 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).

Construction of Expression Plasmid *pPIC9-xynBS27*

To construct the expression vector, a 570-bp DNA fragment coding the mature xylanase without the predicted signal peptide sequence was amplified from the genomic DNA of *Streptomyces* sp. S27 using the *LA Taq* DNA polymerase with GC buffer (TaKaRa, Japan) and primers F1 (5′-ATTGAATTCCAGACCGTCACCTCGAACCAGAC-3′) and R1 (5′-TATGCGGCCGCT CAGCTCACCGTGATGTTGGAGCTTC-3′). The underlined sequences correspond to the *Eco*RI and *Not*I restriction sites, respectively. The PCR cycle condition consisted of an initial step of 5 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 58 °C, and 50 s at 72 °C, and a final extension step of 7 min at 72 °C. The PCR products were purified from the agarose gel, digested with *Eco*RI and *Not*I, and cloned into the pPIC9 vector using these restriction sites to generate the recombinant plasmid *pPIC9-xynBS27*.

Xylanase Gene Transformation and Expression

Competent cell preparation and electroporation were performed according to the instructions included in the *Pichia* Expression Kit manual. The plasmid *pPIC9-xynBS27* was linearized by digestion with *Bgl*II and then transformed into *P. pastoris* GS115 by electroporation using the MicroPulser (Bio-Rad, USA) at 2,000 V with a 0.2-cm cuvette. Then, 1 ml ice-cold sorbitol solution (1 mM) was immediately added to the cuvette, and the contents were spread on RDB plates and incubated at 30 °C for 3 days.

The clones grown on the RDB medium were transferred to MM and MD plates at 30 °C for 2 to 3 days until colonies appeared and then inoculated into 3 ml BMGY medium for 48 h. The cells were collected by centrifugation and suspended in 1 ml BMMY medium. The cultures were then incubated at 30 °C for 48 h with constant shaking (250 rpm). The culture supernatants were harvested by centrifugation (5,300×*g*, 10 min, 4 °C) for xylanase activity analysis. The protein content was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 5% polyacrylamide stacking gel and a 12% separating gel. After electrophoresis, proteins were stained with 0.2% Coomassie brilliant blue R-250.

Xylanase Activity Assay

Xylanase activity was measured using the 3,5-dinitrosalicylic acid method (DNS) [11] with slight modifications. The standard reaction system consisting of 0.1 ml appropriately

diluted enzyme and 0.9 ml McIlvaine buffer (0.2 M Na_2HPO_4/0.1 M citric acid, pH 6.5) containing 1% (*w*/*v*) oat spelt xylan was incubated at 65 °C for 10 min and terminated by adding 1.5 ml DNS reagent. 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.

High-Cell-Density Fermentation of Recombinant *P. pastoris*

The high-cell-density fermentation of recombinant *P. pastoris* was carried out in a 3.7-L fermenter (Bioengineering KLF 2000, Switzerland). The positive colony with the highest xylanase activity screened in shake flask was inoculated into 40 ml yeast peptone dextrose (YPD) medium and grown at 30 °C for 48 h to obtain preculture I. Preculture I was then transferred into 200 ml YPD medium and grown overnight to obtain preculture II, which was then transferred into the 3.7-L fermenter containing 2 L of basal salt medium with PTM1 trace salts solution (*Pichia* Fermentation Process Guidelines, Version B, Invitrogen) and was grown at 30 °C and pH 5.0.

The fermentation process followed the model elucidation protocol with some modifications [12]. After the initial glucose in the fermentation medium was completely consumed, a glucose-fed batch phase was initiated by addition of glucose using a peristaltic pump to increase the cell biomass under restricted conditions. Subsequently, the mixture containing glucose and methanol was added, allowing the cells to adapt to growing in methanol. The mixture was then replaced with 100% methanol to initiate the methanol fed-batch for induction and expression of the recombinant protein for about 156 h. Every 12 h during the induction and expression phase, xylanase activity in the supernatant was assessed, and proteins in culture supernatants were evaluated by SDS-PAGE as described above.

Purification of Recombinant Xylanase

The cell-free fermentation supernatant was harvested by centrifugation at 10,000×*g* for 10 min at 4 °C and precipitated by 80% ammonium sulfate saturation followed by centrifugation. The precipitate was then suspended in 20 mM Tris–HCl (pH 8.5). After dialysis against the same buffer for 16 h, the dialyzed fraction was loaded onto a HiTrap Q Sepharose XL column (GE Healthcare, Sweden) equilibrated with the same buffer. Elution was done with a linear NaCl concentration gradient (0–1 M) at a flow rate of 3 ml/min. The fractions exhibiting xylanase activity were pooled, concentrated, and stored at 4 °C before use. The protein concentration was assayed by the Bradford method using bovine serum albumin as the standard.

The purified xylanase was deglycosylated using 25 U of endo-β-*N*-acetylglucosaminidase H (Endo H) for 1 h at 37 °C according to the manufacturer's instructions (New England Biolabs, USA). The deglycosylated and untreated (control) enzymes were analyzed by SDS-PAGE. For protein identification, the purified protein band was excised from the SDS-polyacrylamide gel, digested with trypsin, and analyzed by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS). The resulting peptide sequences were compared with the known amino acid sequence of XynBS27.

Enzymatic Characterization

For the pH profile, the purified recombinant enzyme activity was determined at 55 °C in buffers of pH ranging from 3.0 to 12.0; these include the McIlvaine buffer for pH 3.0–8.0,

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 xylanolytic activity under standard conditions.

The optimum temperature of the purified recombinant enzyme was determined at the optimum pH over the temperature range from 20 to 90 °C. For thermal stability studies, the purified enzyme was pre-incubated without substrate in the McIlvaine buffer (pH 6.5) at 60, 65, and 70 °C for various periods, and then the residual activity was assayed under standard conditions.

The K_m, V_{max}, and k_{cat} values for the purified recombinant enzyme were determined in the McIlvaine buffer (pH 6.5) at 65 °C with 1–10 mg/ml oat spelt xylan or birchwood xylan as substrate. The data were plotted according to the Lineweaver-Burk method.

To determine the effects of various metal ions and chemical reagents on the activity of the xylanase, the assay was performed in 0.1 M phosphate buffer (pH 6.5) at 65 °C containing 1 mM or 10 mM of NaCl, KCl, $CaCl_2$, LiCl, $CoCl_2$, $NiSO_4$, $CuSO_4$, $MgSO_4$, $FeCl_3$, $ZnSO_4$, $Pb(CH_3COO)_2$, $FeSO_4$, $AgNO_3$, $HgCl_2$, SDS, ethylenediamine tetraacetic acid (EDTA), or β-mercaptoethanol. The degree of inhibition or activation of enzyme activity was expressed as the percentage against the control sample (the control was assayed without addition of metal irons or chemical reagents).

Analysis of Hydrolysis Product

The reaction mixture containing 5 U purified recombinant enzyme and 150 μg xylan (from oat spelt or birchwood) in 200 μl McIlvaine buffer (pH 6.5) was incubated at 37 °C for 12 h. After hydrolysis, the enzyme was removed from the reaction using the Nanosep Centrifugal 3 K Device (Pall, USA). The products were analyzed by high-performance anion-exchange chromatography (HPAEC) with a model 2500 system from Dionex (USA) [13]. Xylose, xylobiose, and xylotriose were used as standards. The hydrolytic products were quantified on the basis of their own standard curves.

Results

Cloning of the Xylanase Gene *xynBS27*

A 497-bp DNA fragment of xylanase was amplified by PCR using the degenerate primers S11F and S11R that were designed based on the conserved region of GH11 xylanases from *Streptomyces*. The product was cloned into the pGEM-T Easy vector. Based on the partially identified sequence, SF and SR primers were synthesized and used for screening the genomic library of *Streptomyces* sp. S27. Approximately 3,000 recombinants were screened, and one clone (PS27-1608) was found to contain the gene *xynBS27*. After sequencing, one complete ORF of 693 bp was identified.

Sequence Analysis

Analysis using SignalP 3.0 Server showed that XynBS27 consisted of a signal peptide of 41 amino acid residues and a mature xylanase domain of 189 residues with a calculated molecular mass of 21.0 kDa. The deduced amino acid sequence of XynBS27 was compared with the GH11 xylanase sequences available from the GenBank. The closest homolog to

XynBS27 was the xylanase from *Streptomyces* sp. zxy19 (75.9% identity, GenBank accession no. ABY90129), followed by a endo-1,4-beta-xylanase precursor from *Streptomyces* sp. S38 (74.5% identity, CAA67143).

Expression of Recombinant XynBS27 in *P. pastoris*

The gene encoding the mature protein was amplified by PCR and cloned into a pPIC9 vector. Linearized *pPIC9-xynBS27* was transformed into *P. pastoris* GS115 genome. Positive transformants were screened based on the xylanase activity of the culture supernatant.

Among the positive clones, the one that produced the highest xylanase activity was selected for high-yield expression in a 3.7-L fermenter. The expression level of XynBS27 increased with induction time. The XynBS27 activity was measured to be 7,624.0± 394.2 U/ml after induction for 156 h, and the maximal secreted concentration of protein was 2.6±0.2 mg/ml (Fig. 1). SDS-PAGE also showed that the amounts of recombinant xylanase increased corresponding to the longer induction time (Fig. 2a).

Purification of Recombinant XynBS27

Recombinant XynBS27 was purified to electrophoretic homogeneity by anion exchange chromatography. The specific activity of the purified XynBS27 was 3,272.0±308.7 U/mg. The recombinant xylanase contained three main bands with molecular weights of about 27.0–45.0 kDa. These values were higher than the predicted molecular weight (21.0 kDa), suggesting the possible occurrence of glycosylation. By using LC-ESI-MS/MS analysis, three internal peptides, GTVSSDGGTYDIYK, YNQPSVEGTR, and TGGTITTGNHF-DAWSR were identified in the three main bands of the purified protein. These amino acid sequences shared 100% identity with the predicted sequence of XynBS27, indicating the three bands were all the recombinant xylanase. The amino acid sequence showed that there were four deduced *N*-glycosylation sites (Asn-X-Ser/Thr). Endo H treatment resulted in the significant decrease of the molecular weight, suggesting that the bands were XynBS27 with different degrees of glycosylation (Fig. 2b).

Enzyme Characterization

The optimum pH of the purified xylanase was pH 6.5, retaining over 65% of its activity at pH 5.0–8.0 (Fig. 3a). For pH stability, more than 80% of the initial activity was retained after pre-incubating in buffers of pH 4.0–12.0 at 37 °C for 1 h (Fig. 3b).

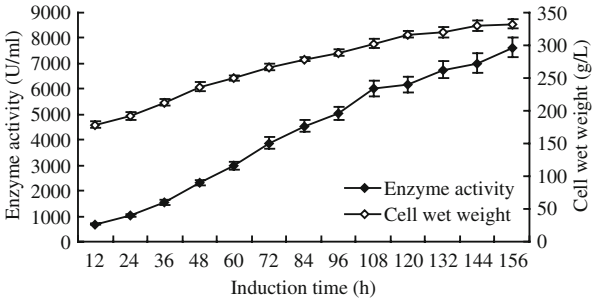

Fig. 1 Accumulation of cell wet weight and enzymatic activity with methanol induction over time in a 3.7-L fermenter

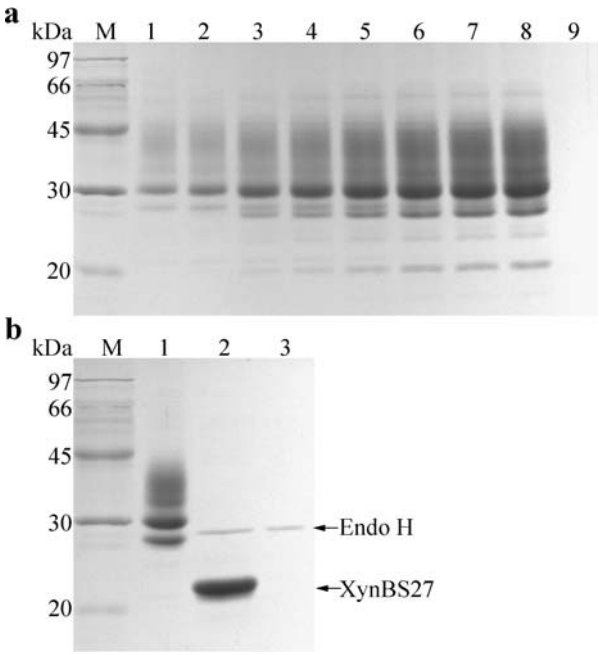

Fig. 2 SDS-PAGE analysis of expression and purification of recombinant XynBS27. **a** SDS-PAGE analysis of XynBS27 expressed in *P. pastoris* with induction over time in fermenter. *M* protein molecular mass standards; *lanes 1–8* culture supernatants after induction of 12, 24, 36, 48, 60, 72, 84, and 96 h, respectively; *lane 9* culture supernatant without induction. **b** SDS-PAGE analysis of the purified and *N*-deglycosylated recombinant XynBS27. *Lane 1* the purified recombinant XynBS27, *lane 2* the *N*-deglycosylated recombinant XynBS27 and Endo H (29 kDa)

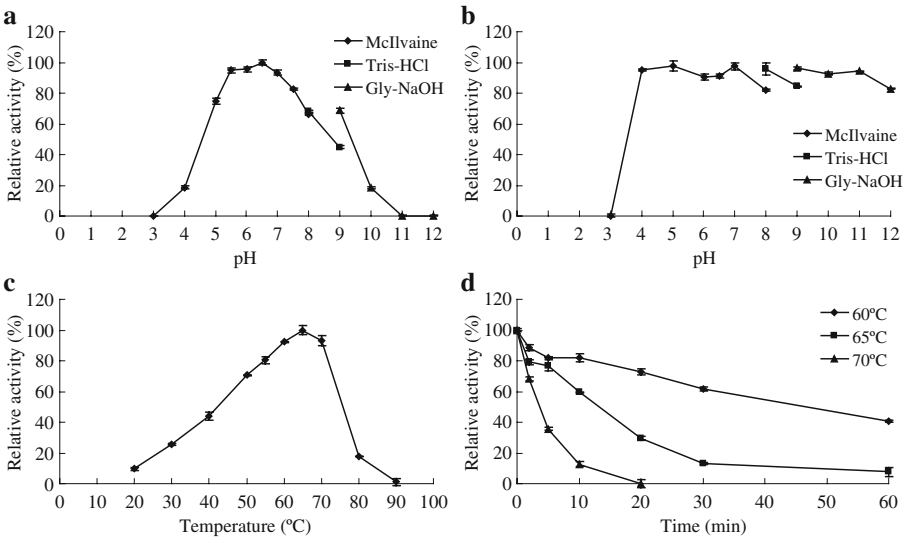

Fig. 3 Characterization of the purified recombinant XynBS27. **a** Effect of pH on xylanase activity. The assay was performed at 55 °C in buffers with pH ranging from 3.0 to 12.0. **b** pH stability of xylanase activity. After pre-incubating the enzyme at 37 °C for 1 h in buffers of pH 3.0–12.0, the residual activity was measured in McIlvaine buffer (pH 6.5) at 65 °C. **c** Effect of temperature on xylanase activity measured in McIlvaine buffer (pH 6.5). **d** Thermostability of recombinant XynBS27. The enzyme was pre-incubated at 60, 65, and 70 °C in McIlvaine buffer (pH 6.5), and aliquots were removed at specific time points for the measurement of residual activity at 65 °C

The purified xylanase displayed maximal activity at 65 °C and lost activity rapidly above 70 °C (Fig. 3c). Concerning thermostability, the enzyme retained 61.6% of its activity after being treated at 60 °C for 30 min, 13.2% when pre-incubated at 65 °C for 30 min, and 68.1% after pre-incubation at 70 °C for 2 min (Fig. 3d).

The xylanase activity of the recombinant XynBS27 in the presence of different metal ions or chemical reagents is shown in Table 1. The activity was absolutely inhibited by Hg^{2+} and strongly inhibited by Ag^{+} even at the concentration of 1 mM. The activities of the enzyme were not affected by most of the metal ions and reagents under test (1 mM). Partial inhibition was observed in the presence of some metal ions and reagents at 10 mM concentration; SDS, Cu^{2+}, Pb^{2+}, and Fe^{2+} reduced the enzyme activity by 59.2%, 49.6%, 32.9%, and 31.0%, respectively. β-mercaptoethanol enhanced the activity about 1.3- and 1.6-fold at the concentrations of 1 and 10 mM, respectively.

Using the double reciprocal plot of Lineweaver and Burk, the K_m, V_{max}, and k_{cat} values using oat spelt xylan as substrate were 6.2±0.5 mg/ml, 9,930.5±450.3 μmol min^{-1} mg^{-1}, and 3,475.7±157.6 s^{-1}, respectively. When birchwood xylan was used as substrate, the values were 2.3±0.2 mg/ml, 4,962.8±254.0 μmol min^{-1} mg^{-1}, and 1,737.0±88.9 s^{-1}, respectively.

Analysis of Hydrolysis Products

The hydrolysis products of oat spelt xylan and birchwood xylan by the purified recombinant XynBS27 were analyzed by HPAEC (Fig. 4). The composition of the hydrolysis products from oat spelt xylan was 14.8±1.1% xylose, 75.2±2.4% xylobiose, and 10.0±0.8% xylotriose. The product composition from birchwood xylan was 15.3±1.2% xylose, 78.9±2.1% xylobiose, and 5.8±0.4% xylotriose.

Table 1 Effect of metal ions and chemical reagents on the activity of XynBS27.

Reagents	Relativity activity (%)	
	1 mM	10 mM
None	100.0±1.2	100.0±1.1
Na^{+}	95.0±0.7	90.2±0.3
K^{+}	95.1±0.4	88.9±0.6
Ca^{2+}	98.1±1.8	90.3±1.7
Li^{+}	94.9±0.8	83.9±2.0
Co^{2+}	95.7±0.6	73.9±3.1
Cr^{3+}	96.3±0.7	116.3±1.9
Ni^{2+}	91.7±3.0	94.7±1.8
Cu^{2+}	86.2±0.5	50.4±1.7
Mg^{2+}	96.2±2.2	103.6±1.1
Fe^{3+}	99.2±0.5	88.7±1.7
Fe^{2+}	105.8±0.9	69.0±1.8
Zn^{2+}	97.6±0.6	92.7±2.0
Pb^{2+}	90.6±2.1	67.1±1.6
Ag^{+}	30.9±1.4	–
Hg^{2+}	0.0	–
SDS	97.5±2.4	40.8±2.9
EDTA	97.6±0.5	96.0±2.9
β-Mercaptoethanol	130.6±1.5	165.4±3.2

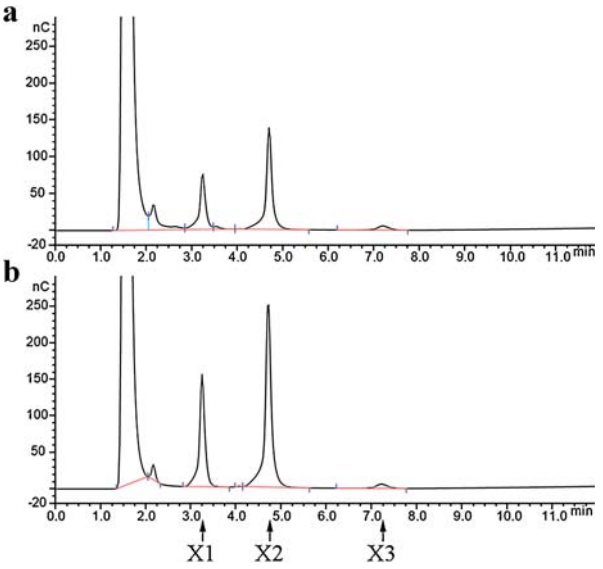

Fig. 4 HPAEC analyses of the hydrolysis products by XynBS27 digestion. **a** The hydrolysis products of oat spelt xylan. **b** The hydrolysis products of birchwood xylan. The positions of standard oligosaccharides, xylose (*X1*), xylobiose (*X2*), and xylotriose (*X3*), are shown by *arrows*

Discussion

Microorganisms belonging to the genus *Streptomyces* have a complex life cycle and produce many valuable commercial enzymes [14]. Several xylanases from *Streptomyces* have been purified and characterized. In this work, the gene *xynBS27* encoding a GH11 xylanase was cloned from *Streptomyces* sp. S27. The xylanase XynBS27 only contains a catalytic domain and does not contain the carbohydrate-binding module or thermostabilizing domains. The two conserved glutamate residues that act as catalytic residues were detected in +127 and +217 of the sequence [15].

The methylotrophic yeast *P. pastoris* has been developed as a high-level expression system because of its advantages for overproduction of heterologous proteins, and a number of active enzymes have been successfully expressed in this system [16, 17]. In this study, XynBS27 was highly expressed and secreted in *P. pastoris* inducible system. Reducing costs associated with xylanase production is one of the major challenges faced today. One approach that has been used to address this problem is to improve xylanase expression through optimizing the codon usage, G+C content, and signal peptide and increasing the gene copy number. The other approach is to choose a xylanase with higher specific activity. The specific activities of XYNB from *S. olivaceoviridis* A1, Xyl1 from *Streptomyces* sp. S38, XynA from *Penicillium* sp. 40, and the xylanase from *S. cyaneus* SN32 were 2,870, 2,100, 1,250, and 894 U/mg, respectively [7, 9, 15, 18]. The specific activity of the purified XynBS27 under this study was 3,272.0 U/mg, which was higher than most *Streptomyces* xylanases. It suggested that XynBS27 could be a good candidate for reducing the cost of xylanase production.

The purified XynBS27 showed the optimum pH of 6.5 and remained active over a very broad pH range of 4.0–10.0. XynBS27 showed over 65% of the maximum activity in the pH ranging from 5.0 to 8.0; it was higher than other *Streptomyces* xylanases of GH11, which exhibited the high sequence homology to XynBS27. For example, STX-II from *Streptomyces thermoviolaceus* OPC-520 and the xylanase B from *S. lividans* showed the optimum pH of 7.0 and 6.5, respectively. But they were almost inactive when assayed at

pH 4.0 or 9.0 and showed about only 55% of the maximum activity in the range of pH 5.0–8.0 [19, 20].

The activity of XynBS27 was partially inhibited by some metal ions and chemical regents at 10 mM concentration. However, Hg^{2+} seemed to be a strong inhibitor since the enzyme activity was completely inhibited when 1 mM Hg^{2+} coexisted. This effect is hypothesized to be due to the fact that Hg^{2+} can oxidize the indole ring, thus interacting with the tryptophan residues of the enzyme. Actually, based on the predicted structure of XynBS27 using SWISS-MODEL, there are seven tryptophan residues in the catalytic domain, and four of them are located near the active sites of XynBS27. In addition, the effects of Cu^{2+} and Fe^{2+} are adverse for xylanase application in industries [21]. However, when 10 mM Cu^{2+} or Fe^{2+} was added to XynBS27, the enzyme retained more than 50% and 69% activity, respectively. These characteristics collectively may facilitate its widespread application. β-Mercaptoethanol could enhance the activity of XynBS27, presumably by counteracting the oxidation effects of the S–S linkage between cysteine residues, thus stabilizing the xylanase [22]. The similar effect has been reported for other xylanases [23].

The hydrolysis products of oat spelt xylan and birchwood xylan by xylanase were a mixture of xylo-oligosaccharides, which have been used as active ingredients of functional foods in many countries [3]. Considering the components of the hydrolysis products, the major product (>75%) was xylobiose. Similar hydrolysis products have been reported for XynC from *Clostridium stercorarium*, XynB from *Thermotoga maritime*, and an endoxylanase from *Bacillus* sp., but they released about 65% of the total hydrolysis products as xylobiose [24–26]. As food ingredients, xylo-oligosaccharides not only can increase the populations of *bifidobacteria* and *lactobacilli*, which constitute important components of the beneficial gut microflora, but also can reduce the concentration of secondary bile acids. Thus, beneficial effects of xylo-oligosaccharides are derived from their ability to suppress the activity of entero putrefactive bacteria, prevent the proliferation of pathogenic intestinal bacteria, facilitate the digestion, and absorb nutrients [3, 27]. However, the production of analytical grade xylobiose is a time-consuming and expensive process [28]. Therefore, our study could provide the basis to develop large-scale production of xylobiose. XynBS27 has several advantageous properties such as high specific activity, good activity over broad pH range, excellent pH stability, and being suitable for xylobiose production. These significant characteristics suggest that XynBS27 may be a good candidate in industrial application.

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