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Cloning and expression of a xylanase *xynB* from *Aspergillus niger* IA-001 in *Pichia pastoris*

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The high-level expression of the xylanase GH11 gene from *Aspergillus niger* IA-001 called *xynB* was successfully completed in *Pichia pastoris*. The *xynB* gene encoding a mature xylanase of 225 amino acid was subcloned into the pPICZαA vector and was transformed into *P. pastoris* X-33 under the control of the alcohol oxidase I (AOX1) promoter. The *xynB* gene was ligated with a sequence encoding modified α-factor signal peptide (pPICZαmA) and the recombinant xylanase activity, which was measured 1280 U ml^{-1}, was 1.5-fold higher than when it was inserted into pPICZαA and was 19.39-fold greater than the native xylanase in the original strain. In a 10 L fermenter, the recombinant xylanase activity measured 10,035 U ml^{-1} after 114 h. The SDS–PAGE analysis revealed that the purified xynB protein migrated as a single band with an apparent molecular weight of 24 kDa. The specific activity, using beechwood xylan as a substrate, was 1916 U mg^{-1}. The xylanase activity was optimal at pH 5.0 and at 50 °C. In addition, the xynB was active over a pH range of 2.2 to 10.0. The apparent K_m and V_{max} values were 4.429 mg ml^{-1} and 1429 U mg^{-1}, respectively.

Keywords: Xylanase / *Aspergillus niger* / *Pichia pastoris* / Expression

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Introduction

Xylan is the major component of hemicellulose [1], which is the second largest recyclable natural resource after cellulose in nature [2]. Xylan is a non-starch-polysaccharide that is in the cell wall and middle lamella of plant cells; it is a complex polysaccharide with a backbone consisting of a β-1,4-D-linked xylose residue [3]. The endoxylanase 1,4-β-D-xylan xylanohydrolase, (EC 3.2.1.8) plays a key role in cleaving the glycosidic bonds and in liberating xylan [4]. Xylanase has potential biotechnological applications in the food and animal feed industry, in the degradation of agricultural wastes, and in the paper bleaching industry [5]. In the animal feed industry, many studies have shown that the xylanase hydrolyzes xylan, reducing the viscosity and improving the nutrient utilization and digestive performance of the feed [6–8]. In Thailand, large amounts of xylanases are used in the animal feed industry every year, especially for the production of chicken and swine feed [9].

Based on sequence similarities and hydrophobic cluster analyses, xylanases have been classified into one of two families of glycosyl hydrolases: family 10 (or F) and family 11 (or G) [3]. Xylanases are widely distributed in nature and are found in plants, algae, insects, protozoans, and microorganisms [9]. *A. niger* is a well-known fungus that produces multiple xylanases with different physicochemical properties [10].

Recently, the methylotrophic yeast *Pichia pastoris* has been characterized as the most suitable yeast host for the expression of eukaryotic proteins [11]. The expression of heterologous proteins in this yeast is driven by the alcohol oxidase I (AOX1), which is induced by methanol and repressed by other carbon sources such as glucose, glycerol, and ethanol [12, 13]. The important features of this heterologous expression system include the ability to stably express heterologous plasmids and the production and secretion of high levels of protein [13, 14].

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In this study, we cloned a xylanase gene from *Aspergillus niger* IA-001, which belongs to glycosyl hydrolase family 11, and was expressed in *P. pastoris* X-33. After fermentation and purification, the specific activity, the optimal conditions, and the stability of recombinant xylanase were determined, suggesting industrial applications and commercial development for the recombinant xylanase. The high level of xylanase activity might increase the yield of enzyme hydrolysis from biomass. Thus, the xylanase could be used as a feed additive for animals.

Materials and methods

Plasmids, microorganisms, and culture conditions

The *A. niger* IA-001 strain (conserved in our laboratory) was cultured in potato dextrose agar (PDA) medium on solid slants at 30 °C. The *E. coli* Top10 was purchased from the Tiangen Co., Ltd (Beijing, China) and was used for the gene cloning and plasmid propagation. The *P. pastoris* X-33 strain (from our laboratory) was used for the heterologous gene expression and was grown in a YPD (2% pepton; 2% dextrose; and 1% yeast extract) medium. The *P. pastoris* transformants were cultured under selective conditions in YPD containing 100 μg ml^{-1} zeocin. The cloning vector pBS-T (Sangon, Shanghai, China) was used for the cloning and sequencing of the PCR fragments. The yeast expression vector pPICZαA (maintained in our laboratory) was used for the heterologous expression of xylanase in *P. pastoris* X-33.

Production of crude xylanase by liquid-state fermentation

The enzyme production was studied in Erlenmeyer flasks (500 ml) containing 300 ml of fluid fermentation medium having (g L^{-1}): wheat bran, 40; glucose, 1; $(NH4)_2SO_4$, 20; Tween 80, 1; KH_2PO_4, 2; $MgSO_4{\cdot}7H_2O$, 0.5. Strains inocula precultures were primed in the 100 ml liquid medium, having 3.0% NaNO3 w/v, 1.0% KH_2PO_4 w/v, 0.5% $MgSO_4$ w/v, 0.5% KCl w/v, 0.01% $FeSO_4$ w/v, and 30% sucrose w/v. Flasks were inoculated with 24 h grown inoculum broth and incubated at 37 °C under shaking condition (220 rpm) in a shaker incubator. The flasks were incubated at 30 °C for 72 h; 5 ml culture contents were centrifuged at 5000 rpm at 4 °C for 10 min. The supernatant was treated as crude enzyme and was assayed for xylanase activity.

Cloning of the *xynB* gene from *A. niger* IA-001

The genomic DNA from *A. niger* was extracted using Bioteke Corporation common plant DNA extraction Trizol (Beijing, China). According to the 5′ and 3′ terminal amino acid sequences of *A. niger* endoxylanases reported in GenBank, two primers (xyl-F and xyl-R) (Table 1) were designed and used for cloning the *xynB* gene. The polymerase chain reaction (PCR) was performed with the PCR mix (Tiangen), using 40 cycles of denaturation at 94 °C for 30 s, annealing at 45 °C for 30 s and extending at 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. The PCR product was purified with a UNIQ-10 DNA purification kit (Tiangen), inserted into the pBS-T plasmid (Sangon), and transformed into *E. coli* Top10 (Tiangen). The transformants (pBS-*xynB*) were screened on LB solid medium (10 g L^{-1} tryptone, 10 g L^{-1} NaCl, 5 g L^{-1} yeast extract, and 15 g L^{-1} agar) and supplemented with 100 μg ml^{-1} ampicillin; the positive recombinant plasmids were sequenced at the Qingke Bioscience Co., Ltd (Beijing).

The xylanase gene without the intron was generated using an overlap PCR amplification procedure. The PCR amplification used the primers xyn-1 and xyn-2 (Table 1) with PCR mix through 5 cycles of 94 °C for 30 s, 45 °C for 30 s, and 72 °C for 1 min, 30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min, and finally 72 °C extension for 10 min. The other PCR product was amplified using two additional primers (xyl-3 and xyl-4) (Table 1) and employing the method described above. Based on these two DNA fragments, the primers (xyl-1 and xyl-4) (Table 1) were used to amplify the sequence of the xylanase gene. PCR amplification was performed as follows: 94 °C for 3 min, 30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. The resulting PCR product was purified using a UNIQ-10 DNA purification kit and ligated into the pBS-T plasmid and was transformed into *E. coli* Top10 to screen the recombinant cloning plasmid pBS-*xynB* without the intron. The plasmid was verified and identified using restriction analysis and DNA sequencing (Beijing Qingke Biological Co., Ltd, China).

Table 1. Primers used in this study.

Name	Sequences (5′-3′)[a]	Size (bp)
xyl-F	ATGCTCACCAAGAACCTT	18
xyl-R	TYACTGAACAGTGATGGA	18
xyn-1	CG**GAATTC**GTTCCCCACGACTCTGT	25
xyn-2	GCGCAGGACATTACCTACAGCGGC	24
xyn-3	AGGTAATGTCCTGCACTTCAAGGGTTC	27
xyn-4	GC**TCTAGA**TCACTGAACAGTGATGGAG	27
xynB-F	CG**GAATTC**GTTCCCCACGACTCTGTCGT	28
xynB-R	GC**TCTAGA**TCACTGAACAGTGATGGAG	27

[a]Restriction sites are underlined and shown in bold.

Construction of the plasmid for xylanase expression and secretion

A pair of specific primers (xynB-F and xynB-R) (Table 1) were designed to amplify the mature protein coding gene without the intron. The amplification was performed using an LA Taq™ DNA polymerase (TaKaRa, Japan) under the following conditions: the first step was at 94 °C for 3 min, followed by five cycles of 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 1 min; the second step was 30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. The PCR product was purified using a UNIQ-10 DNA purification kit, double digested using *EcoR* I and *Xba* I (TaKaRa, Japan), and cloned into the pPICZαA and pPICZαmA vectors, which were digested with *EcoR* I and *Xba* I. In pPICZαmA, the signal peptide sequence was optimized as previously reported [15]. After being transformed into *E. coli* Top10, the recombinant plasmids pPICZαA-*xynB* and pPICZαmA-*xynB* were selected on low salt LB agar plates containing 25 μg ml^{-1} zeocin. The correction of the insert was verified by restriction endonuclease digestion and sequencing as described above.

Transformation and expression of *xynB* gene in *P. pastoris*

The recombinant plasmids were linearized using *Bgl* II (TaKaRa, Japan) and transformed into *P. pastoris* X-33 by electroporation (2.5 kV, 25 μF, 200 Ω, and 4 ms). The transformed cells were cultured at 28 °C on YPDS agar medium (YPD agar medium supplemented with 1 mol/L sorbitol) supplemented with zeocin (100 μg ml^{-1}) for 2–3 days. The yeasts X-33/pPICZαA-*xynB* and X-33/pPICZαmA-*xynB* were cultured at 28 °C and 250 rpm for 24 h in 20 ml of BMGY medium, which consisted of (per L) 13.4 g YNB, 4×10^{-4} g biotin, 10 ml glycerol, 10 g yeast extract, 20 g peptone and 100 mM phosphate, and had a pH of 6.0. The cells were harvested by centrifugation and resuspended in 20 ml BMMY medium [BMGY medium in which the glycerol was replaced with methanol (5 ml L^{-1})] in a 150 ml Erlenmeyer flask kept consistent for 72 h. For the induction, the methanol (0.5%) was added to the culture every 24 h, and the samples were withdrawn at intervals. The cells were collected by centrifugation (12,000 rpm for 1 min), and the supernatant was assayed for xylanase activity.

Recombinant yeast X-33/pPICZαmA-*xynB* in the fermentation

The recombinant yeast X-33/pPICZαmA-*xynB* was incubated in 10 ml YPD medium at 250 rpm and 28 °C overnight. The culture was transferred to 100 ml and was incubated at 250 rpm and 28 °C to an OD600 nm measured 2.35. The culture was scaled up by transferring to a 10 L fermenter containing 3 L sterilized fermentation medium (15 g KH_2PO_4, 2.79 g $CaSO_4$, 54.6 g K_2SO_4, 44.7 g $MgSO_4$, 150 g $NH_4H_2PO_4$, 4.5 g KOH, 150 g dextrose). The fermentations were initiated in batch culture at 28 °C. pH was maintained at 5.0 automatically using ammonia and dissolved oxygen (DO) level was maintained over 30%. When the cellular wet weight yield reached 80–100 mg ml^{-1}, continuous glycerol feeding was carried out after glucose was exhausted (about 36 h). Methanol was fed at 10 ml L^{-1} h^{-1} to induce xylanase expression when all the glycerol was consumed (approximately 4–5 days), and the yield of wet weight reached 220 mg ml^{-1}. The condition of fermentation was performed according to a previously published method [16]. The culture samples were collected every 12–24 h and the cellular wet weight, the enzyme activity and the protein production using SDS–PAGE (12% gels) were determined.

Purification of recombinant xylanase

The cell-free supernatant from the fermentation was recovered after centrifugation at 5000 rpm for 10 min at 4 °C and was treated with ammonium sulfate to 70% saturation to precipitate the enzymes. The precipitated enzymes were centrifuged at 10,000 rpm for 30 min at 4 °C. The precipitate was resuspended in 50 mM PBS (14.51 g L^{-1} $Na_2HPO_4{\cdot}12H_2O$, 1.48 g L^{-1} $NaH_2PO_4{\cdot}2H_2O$, and 7.25 g L^{-1} NaCl), pH 7.4 (buffer A), and was dialyzed against the same buffer at 4 °C overnight using Snakeskin™ pleated dialysis tubing (Pierce, USA). The dialysate (10 ml) was loaded onto an anion-exchange column (DEAE-Sephadex A-50, 2.6 cm × 20 cm, Amersham Biosciences, HK) equilibrated with buffer A. The proteins were eluted from the column using a 0–1 M linear gradient of NaCl in buffer A. The majority of the xylanase activity was eluted as a single peak, and those fractions were pooled and were analyzed using SDS–PAGE.

Xylanase activity assays and protein assays

The xylanase activity was determined by measuring the release of reducing sugar from beech xylan, using the method described by Deng et al. [10]. Four-hundred microliters beech xylan (Sigma X452-100G) at a concentration of 0.8% w/v and diluted in 0.1 M citric acid and 0.2 M Na_2HPO_4; pH 5.0 (buffer B) was added to a 5 ml burette, which was placed in a water bath for 5 min at 50 °C. Next, 400 μl of the crude enzyme diluted in buffer B was added (400 μl buffer B alone was used for the control), and the mixtures were incubated at 50 °C for 20 min. The amount of reducing sugar released into the solution was determined using the dinitrosalicylic acid

method [17], and xylose was used as a standard. The absorbance was measured at 540 nm, and 1 unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol xylose (reducing sugar) per minute under the conditions specified. The analytical measurements were performed at least in triplicate.

The concentration of the purified xylanase using the different purification procedures was determined using the Micro-BCA Protein Assay Kit (Pierce, Rockford, USA); bovine serum albumin was used as the standard. The SDS–PAGE was performed using 12% gels, and the isolated proteins were visualized by staining with Coomassie Brilliant Blue R-250 (Sigma, St. Louis, MO, USA).

Deglycosylation analysis

Non-inactivating deglycosylated analysis: The purified recombinant xylanase (10 μg) was deglycosylated by the addition of 2 μl Endo H (New England Biolabs) for 1 h at 37 °C, according to the manufacturer's instructions. The deglycosylated and untreated xylanase were analyzed using SDS–PAGE.

Inactivation and deglycosylation analysis: The purified recombinant xylanase (10 μg) was combined with 10× glycoprotein denaturing buffer and incubated at 100 °C for 10 min. Next, 2 μl of 10× G5 reaction buffer (New England Biolabs) and 2 μl Endo H were added, and incubated at 37 °C for 1 h.

Characterization and kinetics of xylanase

The optimal pH for enzyme activity of the purified xylanase was determined at 50 °C in 0.1 M citric acid and 0.2 M Na_2HPO_4 buffers with pHs ranging from 2.2 to 7.0. To determine the pH stability, the xylanase was incubated in various pH buffers (0.1 M citric acid and 0.2 M Na_2HPO_4 buffer for pH 2.2–8.0, and 0.2 M Glycine-0.2 M NaOH buffer for pH 9.0–10.0) for 1 h, and the residual xylanase activities were measured using standard assay conditions.

The optimal temperature of the xylanase activity was measured at pH 5.0 (buffer B) for 20 min at temperatures ranging from 30 to 70 °C. The xylanase was incubated in a water bath at 60, 70, or 80 °C for 30 min. The samples were collected every 5 min, and the residual activity was measured using standard assay methods.

To investigate the effects of different metal ions on the recombinant xylanase activity, 10 mM $CuSO_4$, 10 mM $ZnCl_2$, 10 mM $CaCl_2$, 10 mM $FeSO_4$, 10 mM NaCl, 10 mM $HgCl_2$, 10 mM $MgCl_2$, 10 mM $CoCl_2$, 10 mM $MnSO_4$, or 10 mM EDTA were added to the reaction solution, and the xylanase activities were determined using the standard assay condition. The reaction mixtures without metal ions were used as control.

The K_m and V_{max} for the xylanase were calculated using the Lineweaver–Burk plots derived from the activity assay performed at 50 °C in buffer B using 0.5–4 mg ml^{-1} beech xylan as the substrate.

Nucleotide sequence accession number

The nucleotide sequence of xylanase B gene (*xynB*) was deposited into the GenBank database under accession number JX560731.

Results

Cloning and sequence analysis of the *xynB* gene from *A. niger* IA-001

The *xynB* gene encoding the xylanase was amplified, and a 745-bp PCR product containing a single 67-bp intron was generated according to the comparison between the *xynB* and the xylanase cDNA sequences reported in GenBank. Overlap PCR amplification was performed to remove the intron (Fig. 1) using primers based on the sequences at each end of the intron, and the recombinant plasmid pBS-xynB without the intron was obtained. The mature protein coding gene *xynB* comprises 678 nucleotides, and the molar C + G content in the coding region is 57.7%. The *xynB* gene encoded a 225 amino-acid protein with a calculated molecular weight of 24 kDa; the enzyme belongs to glycoside hydrolases family 11. xynB demonstrated high similarity to those xylanase genes reported in GenBank (Fig. 2).

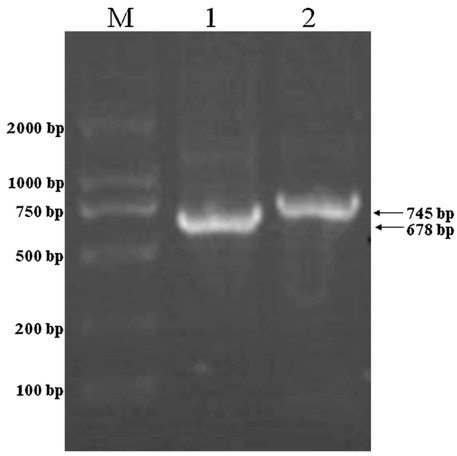

Figure 1. The electrophorosis result of PCR product for xylanase xynB gene. Lane M: DNA 2000L maker. 1: PCR amplification results of xynB cDNA. 2: PCR amplification results of *xynB* as a control. Line 1 is <2 so that the intron has been removed. A 745-bp PCR product contains a single 67 bp intron and the fragment-encoded amino acids are 678 bp. The PCR product was detected by 1% agarose gel electrophoresis, at about 678 bp with a special light-specific amplified band, consistent with the theoretical size.

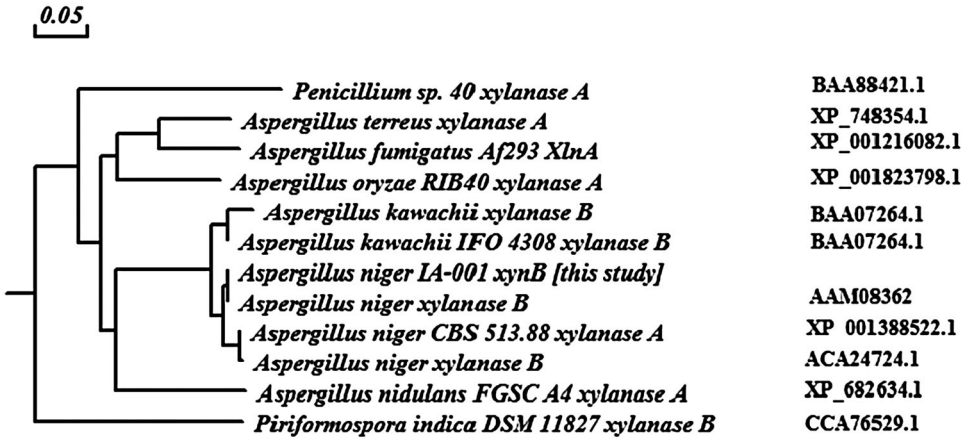

Figure 2. Homology tree based on Amino acids sequences shows phylogenetic relationships between our *xynB* xylanase and homologous xylanase genes. Accession numbers are given behind each species name.

Expression of *xynB* in *P. pastoris*

The mature protein coding gene *xynB* without its intron was ligated into the eukaryotic expression vectors pPICZαA and pPICZαmA, respectively. The constructed plasmids were transfered into *E. coli* Top10 and were isolated, linearized, and transformed into *P. pastoris* X-33. The strains were cultured in BMGY and BMMY, and the activities of the recombinant xylanase were measured after induction at 72 h. The activity of X-33/pPICZαmA-*xynB* was 1280 U ml^{-1} at 72 h, representing a 1.5-fold increase, compared with the X-33/pPICZαmA-*xynB* and 19.39-fold of the original strains.

High-cell-density fermentation

Within 24 h after inoculation, the culture reached a cellular wet weight of approximately 86 mg ml^{-1}; 36 h after the glycerol was added, it was consumed, and the yield of the cellular wet weight reached 210 mg ml^{-1}. The induction phase was initiated by feeding with methanol. The xylanase activity was 844 U ml^{-1} at 6 h after the induction phase. After 114 h of induction, the cellular wet weight reached 340 mg ml^{-1}, and the xylanase activity of the culture medium was 10,035 U ml^{-1} (Fig. 3A). The fermentation supernatant was analyzed using 12% SDS–PAGE (Fig. 3B).

Purification, specific activity, deglycosylation, and SDS–PAGE analysis of the recombinant xylanase

The specific activity of the purified xylanase enzyme was 1916 U mg^{-1}, and the protein content was 6.47 mg ml^{-1}. The SDS–PAGE demonstrated that the recombinant xylanase migrated as a single band with a molecular weight of 24 kDa (Fig. 4A).

The recombinant xylanase was inactivated and treated with Endo H. SDS–PAGE analysis, which demonstrated that molecular weight did not change after the inactivation and deglycosylation of the recombinant xylanase (Fig. 4B). Therefore, the xynB protein is not glycosylated.

Characterization and kinetics of then recombinant xylanase

The optimal pH of xynB activity at 50 °C was pH 5; it maintained more than 88% of its maximal activity after 2 h at a wide range of pHs (from pH 2.2 to 10.0) (Fig. 5A). The optimal temperature of the enzyme at pH 5 was 50 °C (Fig. 5B). However, the thermal stability of xynB was low, and had a residual activity of <40% after incubation of 30 min at 60, 70, and 80 °C. Only 60% activity remained after 5 min of incubation at 80 °C. No activity was observed after 30 min at 80 °C (Fig. 5C).

The effects of different metal ions and chemical reagents on xynB activity was evaluated (Table 2). At 50 °C, the xynB activity was increased by 103.00–132.48% in the presence of 10 mM Co^{2+}, Ca^{2+}, Mg^{2+}, Na^{+}, or EDTA metal ions. However, in the presence of 10 mM Hg^{2+}, Fe^{2+}, or Mn^{2+} metal ions, the enzyme activity decreased slightly to between 93.79 and 63.76%. Most salts, such as 10 mM Cu^{2+} or Ca^{2+}, did not affect the xylanase activity.

The kinetics of xynB were obtained (Fig. 5D). 0.5–4 mg ml^{-1} beechwood xylan was used as a substrate. The assay revealed that the K_m and V_{max} values of the xylanase were 4.429 mg ml^{-1} and 1429 U mg^{-1}, respectively.

Discussion

The *xynB* gene was isolated using a PCR approach. In addition, based on the sequence alignment date, the xylanase gene of mature form encoded 225 amino acids

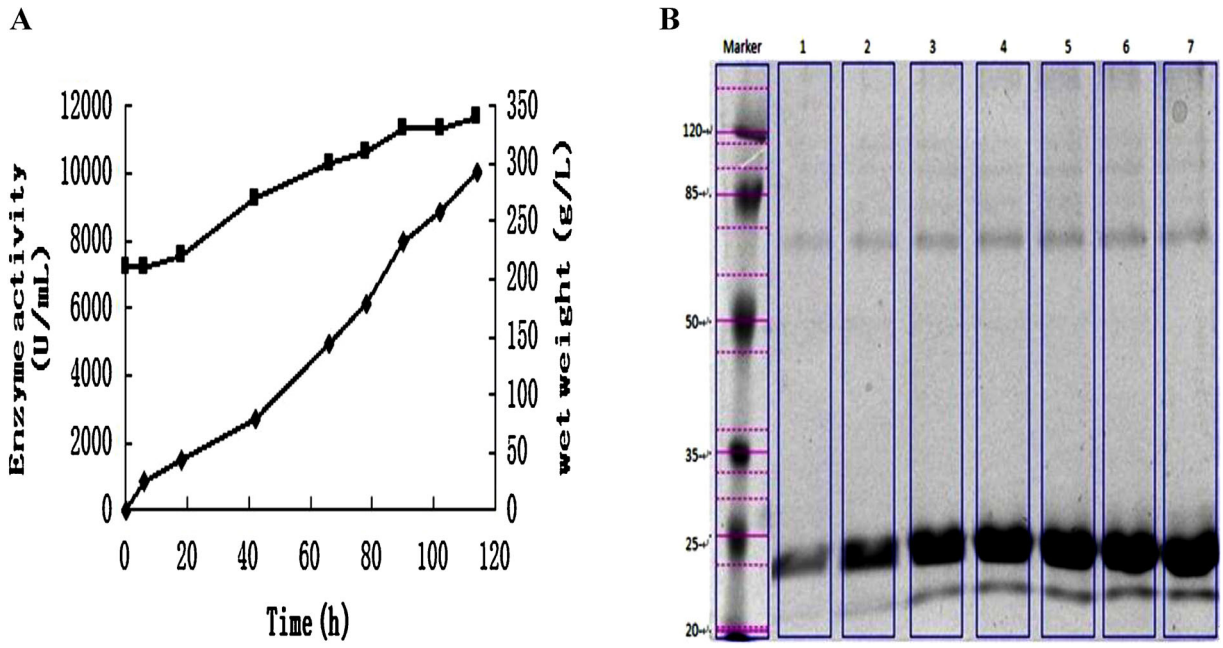

Figure 3. Fermentation of X-33/pPICZαmA-*xynB* in 10 L fermentor. (A) Fermentation process of recombinant yeast in 10 L fermentor. Boxes: Enzyme activity; Lozenges: Biomass. (B) SDS–PAGE analysis of fermentation supernatant. Marker: Protein molecular weight standards; The lane headings 1–7: Expression of the recombinant strain for 6, 18, 42, 54, 66, 90, and 114 h indicate the total induction time in hours.

with an estimated molecular weight of 24 kDa. To date, many xylanase genes have been cloned from varying microorganisms, including *alkaliphilic Bacillus firmus* [18], *Paenibacillus curdlanolyticus* MP-1 [19], *Streptomyces olivaceoviridis* A1 [20], *A. niger* BCC14405 [21]. In this study, the xylanase *xynB* gene was cloned from *A. niger* IA-001 and the amino acid sequence shared 100% similarity with the accession number of AAM08362.1 (Fig. 2).

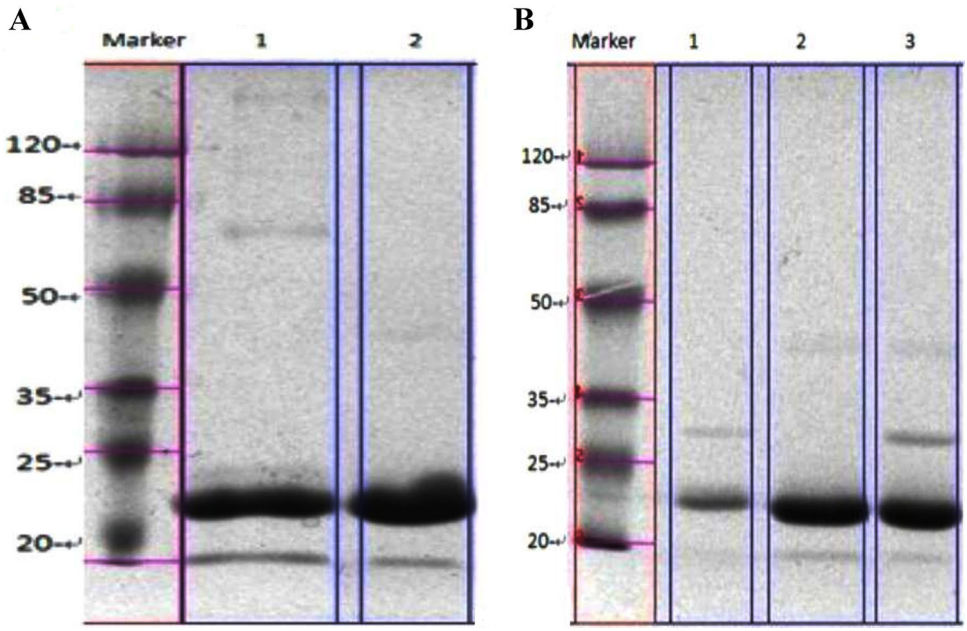

Figure 4. SDS–PAGE analysis of purified recombinant xylanase and xylanase after deglycosylation. (A) SDS–PAGE analysis of purified recombinant xylanase. Marker: Protein molecular weight standards; 1: not purified xylanase; 2: purified xylanase. (B) SDS–PAGE analysis of purified xylanases after deglycosylation. Marker: Protein molecular weight standards; 1: Denaturation xylanases with Endo H; 2: purified xylanases as a control; 3: undenatured xylanases with Endo H.

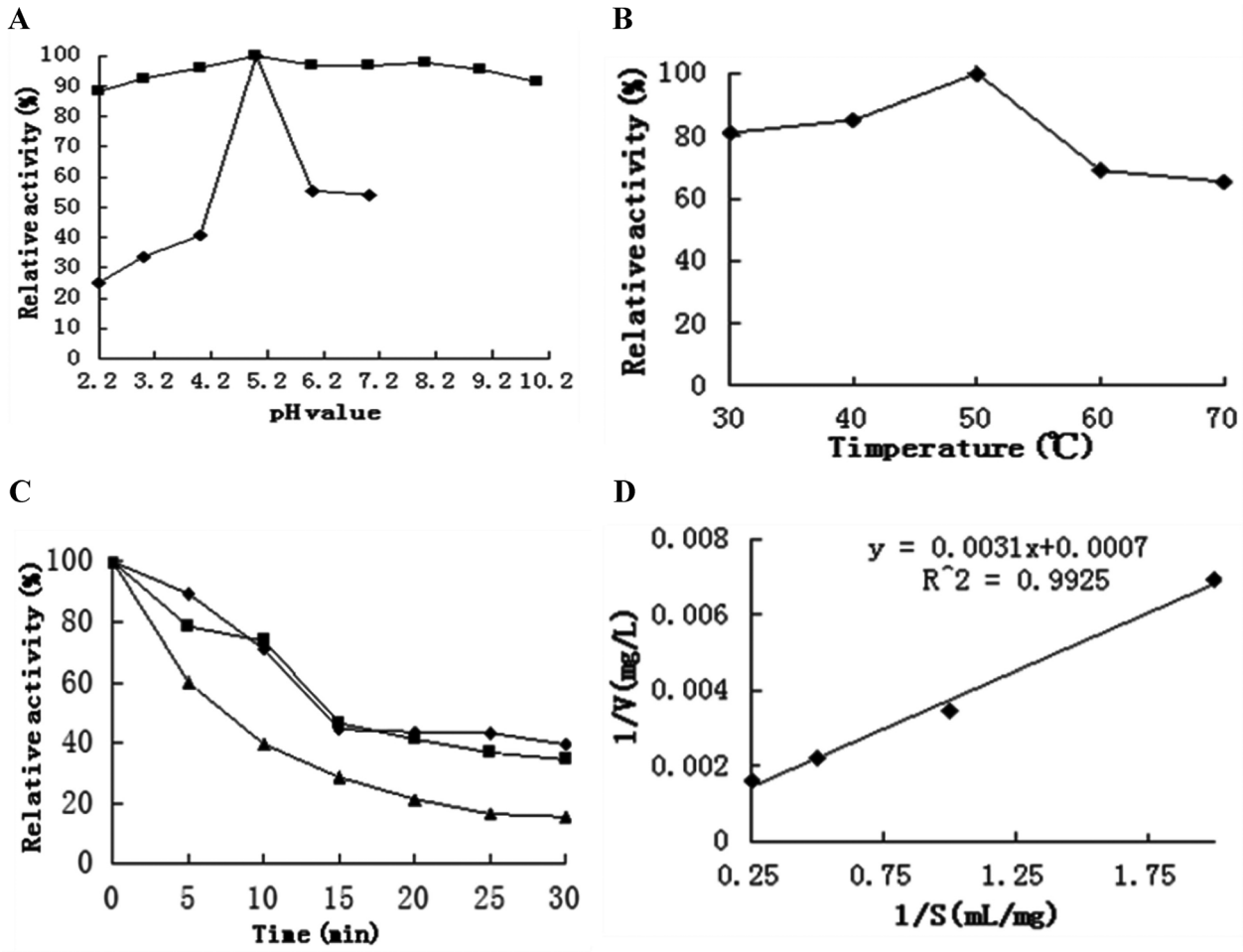

Figure 5. Characterization and kinetics of purified xylanase. (A) Optimal pH and pH stability of xylanase. Boxes: pH stability; Lozenges: Optimal pH. (B) Optimal temperature of xylanase. C: Temperature stability of xylanase at 60, 70, and 80 °C. Lozenges: 60 °C; Boxes: 70 °C; Triangles: 80 °C. (D) Kinetics of xylanase. Lineweaver–Burk double-reciprocal analysis of xylanase with beechwood xylan as the substrate. The enzyme activity was measured with various concentrations of beechwood xylan in buffer A at 50 °C.

Table 2. Effect of metal ions (10 mM) on the activity of the purified xylanase.

Reagents (10 mM)	Relative xylanase activity (%)
Control	100
$MnSO_4$	99.79
$CaCl_2$	103.00
$ZnCl_2$	97.91
$FeSO_4$	85.40
$MgCl_2$	132.48
$HgCl_2$	63.76
$CoCl_2$	106.38
$CuSO_4$	100.37
EDTA	108.19
NaCl	107.03

Generally, the production levels of enzymes in microorganisms is low, and the conditions required for the growth of many microorganisms are incompatible with standard industrial fermentations and downstream processing plants [22]. Therefore, most applications for enzymes depend on their heterologous expression. To date, *P. pastoris* has been developed as an excellent host for heterologous protein expression because it expresses high levels of heterologous proteins both intracellularly and extracellularly [23]. Rakestraw et al. [15] demonstrated one of the isolated mutants in α-mating factor could improve recombinant protein production in *Saccharomyces cerevisiae*. Ruiz et al. [24] reported enhancement of protein secretion in *P. Pastoris* by a modified signal

peptide, but whether mutants in signal peptide could enhance xylanase secretion has not been reported. In this study, the coding sequence of mutant appS4 [15] was synthesized to replace the wild type α-mating factor in the expression vector pPICZαA, and the resulting plasmid pPICZαmA was used in xylanase expression, parallel to pPICZαA. The enzyme activity level of the recombinant X-33/pPICZαmA-*xynB* in shake-flask cultures was 1280 U ml^{-1} under optimal conditions, which was enhanced 1.5-fold using a modified natural signal peptide and was also remarkably higher than that of the A gene (xyl11) encoding xylanase from *Thermobifida fusca* NTU22 (14.5-fold) [25], xynB from *A. niger* (34-fold) [26], the recombinant *T. fusca* xylanase A (11-fold) [27], and XAn11 from *A. niger* US368 strain (1.6-fold) [28].

Due to the improvement of enzyme activity, the X-33/pPICZαmA-*xynB* was fermented, and the activity of the purified recombinant xylanase was approximately 194-fold higher than that obtained in the native *A. niger*. The SDS–PAGE analysis indicated that the recombinant xylanase was expressed at high levels (Fig. 3A), and the expression level and purification strategy was appropriate for the industrial production of the enzyme [29]. The protein content was 6.47 mg ml^{-1}, and the specific activity of purified *xynB* xylanase was 1916 U mg^{-1}, which was approximately 1.5-fold higher than xylanase B in *A. niger* (1250 U mg^{-1}) [30]. Therefore, the biomass can be achieved using high-density fermentation technology, demonstrating the potential of producing recombinant xylanase commercially. The fact that the SDS–PAGE analysis of the xylanase was deglycosylated demonstrated that the xynB protein is not glycosylated (Fig. 4B), which is in contrast to a report by Mclntosh et al. [31]. The purified xylanase exhibited maximal activity at 50 °C and pH 5.0, and it efficiently hydrolyzed beech xylan in the pH range of 2.2–10.0 at 50 °C (approximately 88% of the activity remained at pH 2.2 after incubation at room temperature for 2 h). The date demonstrates that the xylanase is weakly acidic, which is consistent with other reports [32–34], suggesting that the xylanase is a mesophilic enzyme [35]. The K_m values of xylanases are typically between 0.5 and 5 mg ml^{-1} [5], and the *xynB* gene showed rational value of 4.429 mg ml^{-1}, which is similar to the β-xylanase gene from *Trichoderma reesei* Rut C-30 of 4.1 mg ml^{-1} [30] and 4.8 mg ml^{-1} of *A. niger* xylanase A [22]. The V_{max} value of the xylanase was 1429 U mg^{-1} and four times higher than V_{max} which is 325 ± 41 U mg^{-1} [36]. In addition, various metal ions have different effects on the xylanase activity (Table 2). The strong (almost 63%) inhibition by Hg^{2+} is consistent with the report by Cevher and cowokers [37]. The inhibitory effect of metal ions on xylanase activity indicates that the xylanase might contain a metal-binding site near the catalytic region, as previously reported for many xylanases that are inhibited to different degrees by various metal ions [30–40]. It has been suggested that binding with metal ions causes a conformational change in the xylanase molecule [41]. Mg^{2+} significantly enhanced the xylanase activity (approximately 1.3-fold), suggesting that Mg^{2+} can be added to animal feed.

The properties of the recombinant xylanase make it a candidate for various industrial applications. For example, the broad pH stability and optimal temperature of the enzyme make it suitable for use in the animal feed industry because the pH and temperature of the digestive tract of livestock are approximately pH 4.8 and 40 °C [42].

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