SYNONYMOUS CONDON USAGE BIAS AND OVEREXPRESSION OF A SYNTHETIC *xynB* GENE FROM *Aspergillus niger* NL-1 IN *Pichia pastoris*

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To further improve the expression level of recombinant xylanase in *Pichia pastoris*, the *xynB* gene, encoding the mature peptide from *Aspergillus niger* NL-1, was designed and synthesized based on the synonymous condon bias of *P. pastoris* and optimized G+C content. 155 nucleotides were changed, and the GC content decreased from 57.7% to 43.6%. The synthetic *xynB* was inserted into the pPICZaA and then integrated into *P. pastoris* GS115. The activity of the recombinant xylanase reached 1414.7 U/mL, induced with 0.8% methanol after 14-day cultivation at a temperature of 28°C in shake flasks, which was 267% higher than that of the native gene. Furthermore, the maximum xylanase activity of 20424.2 U/mL was obtained by high-density fermentation in a 5-L fermenter, which was the highest xylanase expression in *P. pastoris* yet reported. The recombinant xylanase had its optimal activity at a pH of 5.0 and temperature of 50°C. The recombinant xylanase was stable over a pH range of 4.5 to 8.0. Thus, this report provides an industrial means to produce the recombinant xylanase in *P. pastoris*.

Keywords: Endoxylanase xynB; Synonymous condon; Synthesis; Over expression; Aspergillus niger

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INTRODUCTION

Lignocellulosic biomass is considered a prime alternative to fossil fuels and a source for many of our fuel and chemical feedstock needs. Xylans, as the most abundant hemicellulose of the plant cell wall, consists of a β-1,4-linked D-xylose polymer with arabinosyl-, acetyl-, and/or 4-0-methylglucurosyl side branches (Li and Ljungdahl 1994), which represent a major renewable carbon resource in nature (Haki and Rakshit 2003). Among xylan-degrading enzymes, endo-1,4-β-D-xylanases (EC 3.2.1.8) are key enzymes for random cleavage of the xylan backbone (Huang *et al*. 2006) and hence have broad industrial significance because they have broad uses and potential applications, including biopulping and biobleaching in the pulp and paper industry, bioconversion of lignocellulose material to fermentative products, feed additive of animals, and use in the brewing industry (Liu and Liu 2008; Qiu *et al*. 2010; Khandeparker and Numan 2008). Therefore, the topic of producing high-activity and low-cost xylanase has become hot in these fields.

Xylanases are mainly produced by bacteria and fungi (Sunna and Antranikian 1997). *Aspergillus niger* is a well known fungus that produces multiple xylanases with

different physicochemical properties (Krengel and Dijkstra 1996). In a previous study conducted in our laboratory, a specific endo-β-1,4-xylanase obtained from *A. niger* NL-1 was identified as an excellent feed additive for broilers (Li *et al*. 2010). However, the application of the xylanase was hampered by the presence of several other enzymes, such as cellulase, β-glucosidase, and β-xylosidase, as well as the products of the enzymes. Large-scale production of recombinant xylanase has been facilitated with the advent of genetic engineering. Genes encoding xylanases have been cloned in homologous and heterologous hosts with the objectives of overproducing the enzyme (Baba *et al*. 1994; Ahmed *et al*. 2009).

In this study, the *xynB* gene was optimized and synthesized to match the codon preference of *Pichia pastoris*, and the recombinant enzyme was characterized. The induction conditions of expression and fermentation strategy were also discussed.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Media

A. niger NL-1 was isolated from a soil sample collected in a suburb of Nanjing for its activity of hemicellulase, which was preserved by our laboratory and was grown at 30°C in YPD medium. *Escherichia coli* TOP10F' and the vector pMD-19T (TaKaRa, Japan) were used for general DNA manipulations and for DNA sequencing. *P. pastoris* GS115 (his4) and the expression vector pPICZαA (Invitrogen, USA) were used for the heterologous expression of the optimized xylanase *xynB*. The recombinant strain, pPICzαA-xynB, was constructed as described in a previously published paper by our laboratory (Li *et al*. 2010).

The DNA purification kit, restriction endonucleases, T4 DNA ligase, and Primerstar DNA polymerase were purchased from TaKaRa. Birchwood xylan was obtained from Sigma Chemical Company (USA). All other chemicals were of analytical grade. GenScript synthesized the primers.

Luria-Bertani (LB) medium, YPDS medium, minimal dextrose (MD) medium, minimal methanol (MM) medium, buffered glycerol complex (BMGY) medium, fermentation basal salts medium (BSM), and PTM1 trace salts solution were prepared according to the manual of Pichia Expression Kit (Invitrogen 2002).

Optimization and Oligonucleotide Design for Synthesis of Xylanase Gene

The gene sequence of xylanase *xynB* obtained by our laboratory (Li *et al*. 2010) was selected and optimized according to codon usage bias of *P. pastoris*, GC content, repeat sequence, and RNA instability motif (http://www.genscript.com.cn/technology). About 28 oligomers, with overlap regions with their neighbors, were designed to synthesize the optimized *xynB* gene based on DNAWorks (Hoover and Lubkowski 2002; Rouillard *et al*. 2004), as shown in Table 1. Overlap melting temperatures were designed to be 55 to 60°C. The P1 and P28 oligomer were designed to contain an *Eco*R I site and an *Xba* I restriction site, respectively.

Table 1 Sequence of Oligomers Used in Synthesis of Codon-Optimized *xynB*

Oligomers sequence (from 5' to 3')	
P1	CCCGAATTCGTTCCTCACGACTCCGTTGTTG
P2	CTTATGCAAAGCATCTGATCTTTCAACAACGGAGTCGTG
P3	AAAGATCAGATGCTTTGCATAAGCTTTCAGAAAGAAGTACAC
P4	TGTTCTCTCCGGTGGAAGATGGTGTACTTCTTTCTGAAAGCTTATG
P5	CATCTTCCACCGGAGAGAACAATGGTTTTTACTATAGTTTCTGGACT
P6	TGTAACGTCACCTCCACCATCAGTCCAGAAACTATAGTAAAAACCAT
P7	GATGGTGGAGGTGACGTTACATACACCAACGGAGATGCTG
P8	ACTCCATTCGACTGTATATGAACCAGCATCTCCGTTGGTGTA
P9	GTTCATATACAGTCGAATGGAGTAACGTTGGTAATTTTGTCGGAG
P10	GAACCTGGGTTCCATCCTTTACCTCCGACAAAATTACCAACGTT
P11	GTAAAGGATGGAACCCAGGTTCCGCCCAAGACATTACTTACTCT
P12	CCGGAAGGTGTGAAAGTTCCAGAGTAAGTAATGTCTTGGGCG
P13	GGAACTTTCACACCTTCCGGAAATGGTTACTTGTCAGTTTATGGT
P14	CGATAAGTGGATCTGTAGTCCAACCATAAACTGACAAGTAACCATTT
P15	TGGACTACAGATCCACTTATCGAATACTACATCGTTGAGAGTTACG
P16	TCCAGAACCAGGATTATAGTCTCCGTAACTCTCAACGATGTAGTATT
P17	GAGACTATAATCCTGGTTCTGGAGGTACTTACAAGGGAACCGTTA
P18	TCGTAGACGGAACCATCAGAAGTAACGGTTCCCTTGTAAGTACC
P19	CTTCTGATGGTTCCGTCTACGACATCTACACAGCCACCAGAAC
P20	CTGGATGGAGGCAGCGTTAGTTCTGGTGGCTGTGTAGATG
P21	TAACGCTGCCTCCATCCAGGGTACAGCAACCTTTACTCAAT
P22	CTCTTATTCTGTCTAACTGACCAATATTGAGTAAAGGTTGCTGTACC
P23	ATTGGTCAGTTAGACAGAATAAGAGAGTTGGAGGTACTGTCACCAC
P24	CCCATGCATTGAAATGGTTAGAAGTGGTGACAGTACCTCCAACT
P25	TTCTAACCATTTCAATGCATGGGCTAAATTGGGAATGAACCTTGGT
P26	GCGACGATTTGATAATTGTGAGTACCAAGGTTCATTCCCAATTTAG
P27	ACTCACAATTATCAAATCGTCGCAACAGAGGGTTACCAAAGTTCA
P28	CCCTCTAGACTACTGAACTGTAATAGAACTACTTCCTGAACTTTGGTAACCCTCTG TT

Synthesis of *xynB* Gene and Construction of the Expression Vector

The 624 bp mature peptide domain of *xynB* was reconstructed in a two-step procedure. The initial primer extension step was performed in 50 μL reaction volumes containing 5 μL oligonucleotides P1 to P28 (100 nM each) mixture, 10 μL 10 × ExTaq DNA polymerase buffer, 2.5 U ExTaq polymerase, and 200 μM each of dNTPs and 2 mM $MgCl_2$ under the following PCR conditions: a hot start at 94°C for 5 min, 18 cycles of 94°C for 30 seconds, 59°C for 30 seconds and 72°C for 50 seconds, followed by one cycle of 72°C for 10 min. To amplify the target fragment, 2 μL of the product resulting from the first PCR was used for the second step with forward primer P1 and the reverse primer P28. The concentration of ExTaq polymerase was as above. The PCR parameters were: denaturation at 94°C for 5 min first; 30 cycles of (30 seconds at 94°C, 30 seconds at 64°C, 50 seconds at 72°C); followed by 10 min at 72°C. The PCR product was gel purified and cloned into pMD-19T vector, and the resulting plasmid pMD-19T-SxynB was then transformed into *E. coli* TOP10F′ cells for sequencing.

For the expression of *syn-xynB* in *P. pastoris*, the synthetic *xynB* sequence was amplified by PCR from the cloning plasmid pMD-19T-SxynB using the primers P1 and P28. The PCR product was gel purified and digested with *Eco*R I and *Xba* I before

cloning into the plasmid pPICZαA vector at restriction sites *Eco*R I and *Xba* I, resulting in the recombinant plasmid pPICZαA-SxynB. After being transformed into *E. coli* TOP10F', the transformants were cultured in LLB medium containing 25 μg/mL Zeocin. The positive recombinant vector containing the *syn-xynB* fragment was confirmed via resequencing.

Expression of *syn-xynB* in *P. pastoris* and Mut Phenotype Selection

The recombinant plasmid pPICZαA-SxynB was linearized using *Bst*X I and transformed into *P. pastoris* GS115 competent cells by electroporation according to the Pichia expression vectors manual (Invitrogen 2002). The transformed cells were selected on the basis of Zeocin resistance using YPDS plates containing 500 μg/mL of this antibiotic at 28°C until colonies appeared. The single colonies were picked and transferred to MM and MD plates, respectively, to identify the Mut phenotype using GS115/Mut^s Ablumin and GS115/pPICZ/lacZ Mut^+ as parallel strains.

The selected clones were tested for expression of xylanase in a shake flask with 30 mL BMGY medium at 28°C with constant shaking at 180 rpm according to the manufacturer instructions (Invitrogen). To maintain induction, 100% methanol was added to the culture to a final concentration of 0.5% (v/v) every 24 hours. The xylanase activity was measured every 24 hours according to standard methods. The recombinant strain with the best expression performance was then used for further studies.

Optimization of Syn-xylanase Expression in BMGY Medium

The recombinant strain was incubated in 10 mL of BMGY medium for 48 hours in 28°C with constant shaking at 180 rpm. When OD_{600} reached between 2 to 6, the cultures were harvested and resuspended in 30 mL of BMGY until an OD_{600} of 1.0 was reached for the shaking culture at 28°C with a constant shaking at 180 rpm. Methanol (100%) was added daily (final concentration 0.5% (v/v)) to maintain induction. The samples were centrifuged at 12,000 rpm for 5 minutes. The supernatant was stored at 4°C to determine enzyme activity.

Maintaining all factors at constant levels, except for the one being studied, the culture medium and culture condition were optimized for xylanase production. The expression at a different initial pH (ranging from 3.0 to 8.0) was set. Induction was continued with the addition of methanol to achieve concentrations ranging from 0.5% to 1.5% (v/v) at every 24 hours to sustain the expression after incubation for 48 hours. The effect of different histidine concentrations (0.05%, 0.1%, 0.2%, 0.3%, and 0.5%) on expression of xylanase was studied.

High-Cell-Density Fermentation in a 5 L Fermenter

Larger scale production was carried out in a 5 L fermenter using BSM medium. A 300 mL inoculum in BMGY medium was used to inoculate the 5 L fermenter of BSM medium with 13 mL PTM1 trace salts solution at 28°C. Before inoculation, the pH was adjusted to 6.0 with concentrated ammonium hydroxide, and 3% histidine, and 18 mL biotin (0.02%) was supplemented. The initial cultivation continued until the glycerol had been consumed (about 27 hours), and was followed by the glycerol-fed phase at a continuous feeding rate of 0.6 mL/min/L and initial fermentation volume of 50% (v/v)

glycerol supplemented with 3% histidine and 12 mL/L glycerol PTM1 trace salts solution until cell density reached 240 g/L wet weight. Before starting the methanol induction phase, the glycerol feed was completely stopped and the dissolved oxygen level kept towards 100% for 2 hours to avoid repression of the AOX promoter. The methanol feeding was started at 3.6 mL/h/L fermentation volume containing 3% histidine and PTM1 trace salts solution (12 mL/L methanol) during the first 2 to 4 hours, and increased up to 7.2 mL/h/L for the following 2 hours. The feeding rate was further increased to 10.9 mL/h/L for the remainder of the fermentation. During the induction period, the pH was kept at 5.0. Pure oxygen was supplemented to keep the DO level above 20% during the whole fermentation phase. Samples were collected every 12 hours for optical density measurement, cellular wet weight, xylanase activity assay, and determination of total soluble protein and SDS-PAGE analysis.

Enzyme and Protein Assays

Xylanase activity was measured using 1% birchwood xylan as a substrate in 50 mM sodium citric acid buffer, pH 5.0, at 50°C, for 30 min. The liberation of reducing sugar was estimated by the dinitrosalicylic acid (DNS) method, using xylose as a standard (Miller 1959). One unit of xylanase activity was defined as the amount of enzyme that liberated 1 μmol of reducing sugar from the substrate solution per minute.

The optimal temperature was determined by the standard activity assay at various temperatures from 30 to 70°C at pH 5.0. To estimate thermal stability, the enzyme was pre-incubated for 30 min at the different temperatures. The optimal pH was determined at 50°C for 10 min in a sodium citrate buffer at a pH range from 3.0 to 8.0. The pH stability of the enzyme was determined by examining the residual activities under standard conditions after a pre-incubation of the enzyme at room temperature for 30 min at various pH levels. The activity of the enzyme without pre-incubation was defined as 100%.

SDS-PAGE was performed in gel containing 12% (w/v) acrylamide and 0.1% SDS (w/v), using a Tris/glycin buffer system. Resolved proteins were visualized by staining with Coomassie Brilliant Blue R-250 (Laemmli 1970). Protein concentrations were determined using the Bradford method with bovine serum albumin as a standard (Bradford 1976).

Hydrolysis Products of Birchwood Xylan

The 1% (w/v) birchwood xylan in pH 5.0 and 50 mM sodium citrate buffer was incubated with syn-xylanase at 45°C. The aliquots were removed and extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1) at different time intervals, and standard xylose was analyzed by HPLC with Sugarpak I column, pure water as the mobile phase (0.5 mL/min), and an injection volume of 10 μL.

Nucleotide Sequence Accession Number

The nucleotide sequence optimized *xynB* genes was deposited in the Genbank database under accession number HQ385274.

RESULTS AND DISCUSSION

Synthesis of Codon-Optimized *xynB* Gene and Construction of the Expression Vector

P. pastoris is an efficient host for recombinant protein production with the advantages including growth to very high cell densities in a simple, defined medium and strongly inducible promoters (Zhang *et al*. 2000). However, the use of synonymous codons may vary widely between different genes and organisms. Like most living organisms, yeasts display usage bias towards codons (Nakamura *et al*. 1999). The presence of a large rare-codon in the heterogeneous gene expressed in *P. pastoris* may lead to compromising the host by depleting charged pools of the rare tRNA, and may lead to ribosome pausing that is deleterious to high levels of expression (Brandes *et al*. 1996). Therefore, the expression levels of *xynB* may be further improved through optimizing for codon usage, G+C content, as well as signal peptide (Teng *et al*. 2007). The optimization of codon coding target protein, by the codon bias of the host cell, can usually result in an average 10- to 50-fold increase of target protein production (Outchkourov *et al*. 2002; Sinclair and Choy 2002). Optimizing the conditions of the fermentation is another method that can increase the quantity of recombinant protein (Wang *et al*. 2007).

It was found that usage biases of codon in *A. niger* and *P. pastoris* have significant differences. The DNA sequence of the *A. niger* NL-1 *xynB* gene showed that some amino acid residues were encoded by codons that are rarely used in *P. pastoris*. To further improve the expression level of recombinant xylanase in *P. pastoris*, the *xynB* gene was optimized based on the codon usage bias of *P. pastoris*. At the same time, the mRNA secondary structure, GC content, and unfavorable peaks were optimized to prolong the half-life of the mRNA. The Stem-Loop structures, which impact ribosomal binding and stability of mRNA, were broken. Moreover, to avoid premature termination, changing AT-rich codons to AT-deficient ones eliminated AT-rich stretches. The synthetic gene sequence, in which a total of 155 nucleotides were changed, had 43.6% GC content compared to 57.7% GC content of the initial gene as shown in Fig 1, closer to the actual GC content in *P. pastoris* (Huang *et al*. 2008). The optimized gene without a signal peptide sequence was synthesized by overlapping extension PCR using 28 oligomers designed by the online program DNAWorks. The mature peptide sequence of the optimized *xynB* gene, with a length of 624 bp, was cloned into pMD-19T and sequenced. Then, the fragment was amplified from the cloning plasmid pMD-19T-SxynB and was introduced into the expression plasmid pPICZαA to yield the recombinant plasmid pPICZαA-SxynB.

Transformation, Screen, and Expression of *syn-xynB* in *P. pastoris*

The recombinant plasmid pPICZαA-SxynB was integrated into *P. pastiris* GS115 strain after being linearized using *Bst*X I by electroporating. About 105 transformants were screened on a YPDS plate. The Mut phenotype was identified in MD and MM medium for the pPICZαA-SxynB recombinant cell. Colonies that grew normally in both of the two plates were of the Mut^+ type; however those that grew normally only in the MD plate but slowly in the MMH plate were of the Mut^S type. The results showed that all the transformants were Mut^+ type. The colonies were selected for induction expression.

After 14 days of induction in a shake flask, the colony with the highest xylanase activity, 1408 U/mL (Fig. 2a), was approximately 3-fold higher than that of the native recombinant (Li *et al*. 2010).

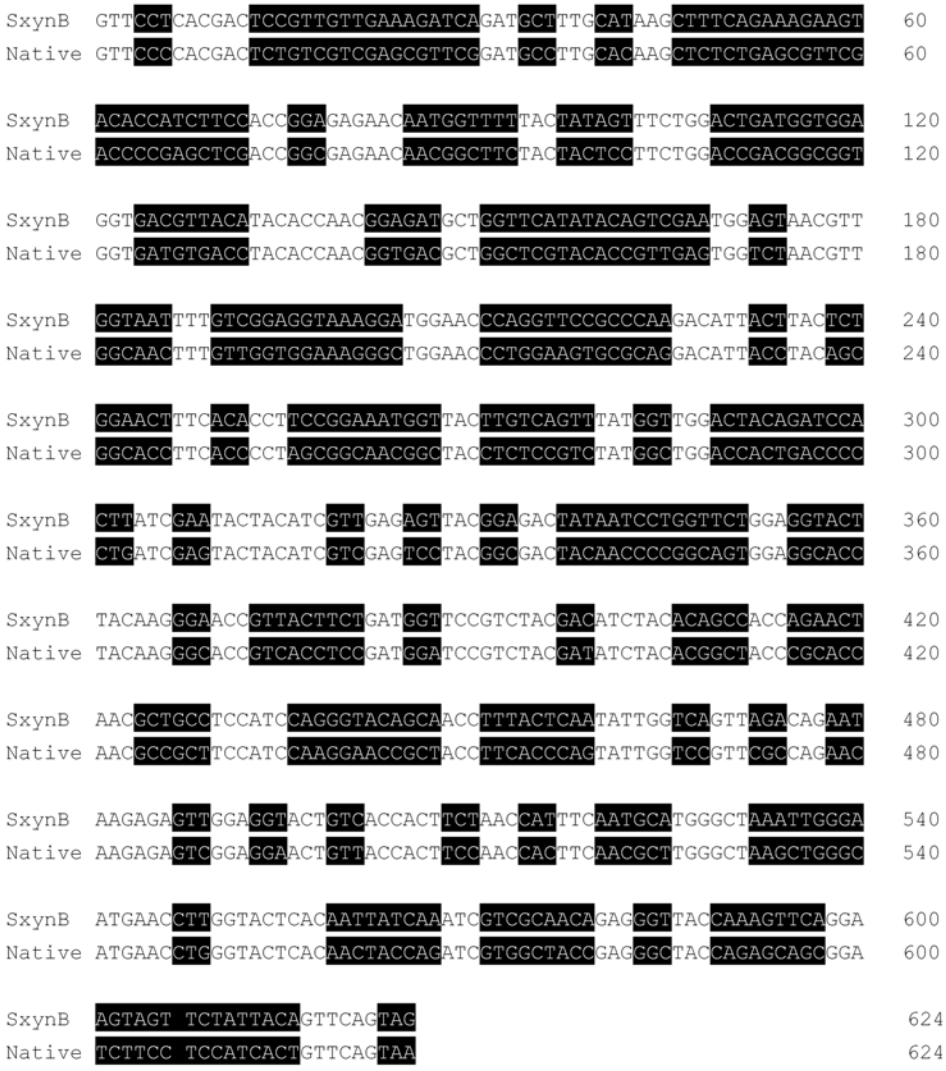

Fig. 1. Alignment of nucleotide sequence between the native and optimized *xynB* gene. The gene encoding 131 residues in *xynB* were optimized. The shadow indicates optimized residues of *xynB*.

Optimization of Syn-xylanase Expression in BMGY Medium

The transformant was used to study the effect of methanol concentration on the xylanase expression. *P. pastoris* is a kind of methylotrophic yeast, capable of metabolizing methanol as its sole carbon source. The promoter regulating the production of alcohol oxidase is the one used to drive heterologous protein expression in *P. pastoris*. So, methanol concentration in a *P. pastoris* process is extremely important (Sreekrishna *et al*. 1997), since high levels of methanol can be toxic to the cells and low levels of methanol may not be enough to initiate transcription. In this study, we can see that the

optimal methanol concentration was 0.8%, with the maximum xylanase activity of 1418 U/mL, as shown in Fig. 2b. When the methanol concentration was above or below 0.8%, the activity decreased significantly.

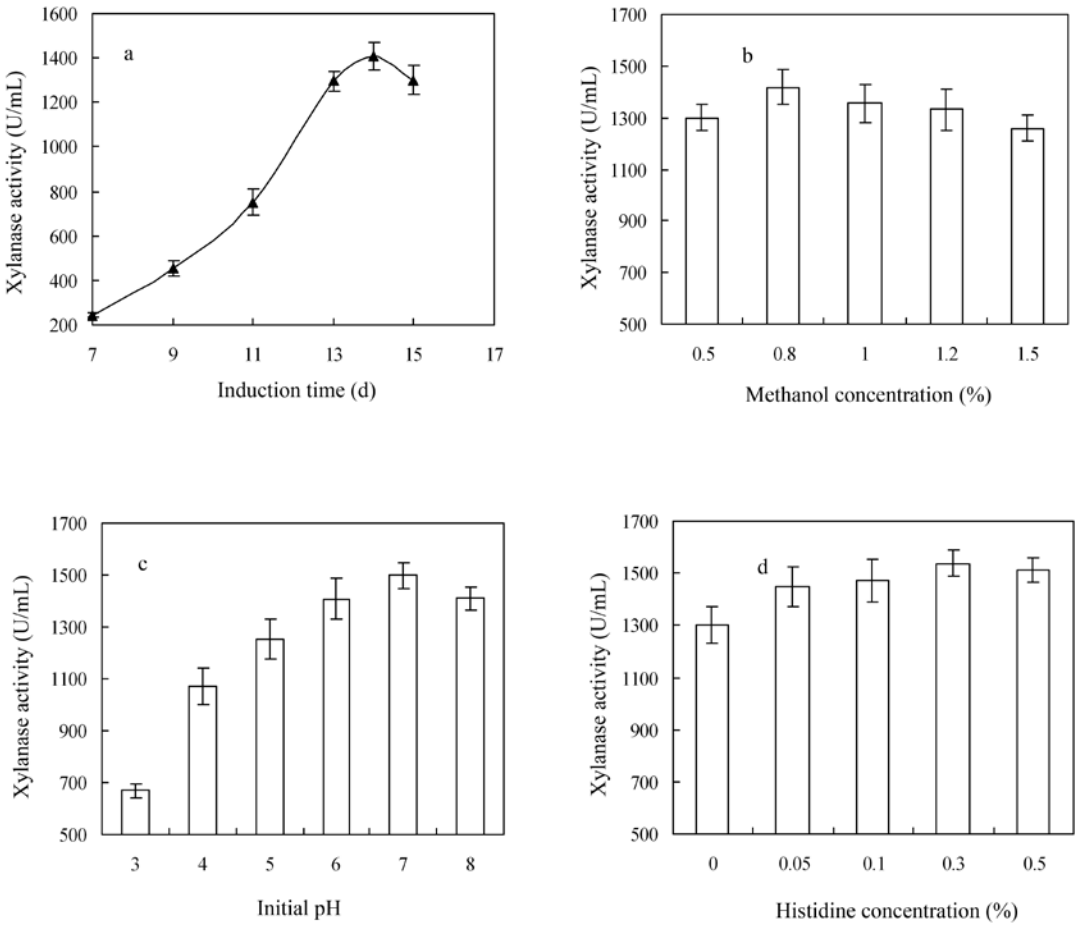

Fig. 2. Expression of recombinant syn-xylanase in *P. pastoris*: (a) Syn-xylanase activity level of the transformant with the highest activity incubated at 28°C, 180 rpm, pH 6.0 in 30 mL BMGY medium. After 24 hours, 0.5% methanol was added every 24 hours to the induction expression. (b) Effect of methanol concentration on xylanase expression. The transformant with the highest activity was induced at pH 6.0 at different methanol concentrations with initial OD_{600} of 1.0. (c) Effect of pH values on xylanase expression. The transformant with the highest activity was induced at a different initial pH with initial OD_{600} of 1.0 and 0.5%, and methanol was added every 24 hours. (d) Effect of histidine concentration on xylanase expression. The transformant with the highest activity was induced at pH 6.0 at different histidine concentrations with initial OD_{600} of 1.0 and 0.5% methanol added every 24 hours with no histidine added as a control. The above mentioned were cultivated and inducted at 28°C, 180 rpm, in 30 mL BMGY medium.

The pH value has an influence on the growth and metabolism of *P. pastoris* in general. To study the effect of pH in the BMGY medium on xylanase expression, we incubated the recombinant in BMGY medium containing 50 mM of K_2HPO_4-KH_2PO_4 buffer. The cultures were prepared with initial pH values of 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0. The results showed that syn-xylanase production increased as the pH values

increased within a pH range between 3.0 and 7.0, but the activity slightly decreased when the pH reached 8.0, as shown in Fig. 2c.

The *P. pastoris* GS115 has a mutation in the histidinol dehydrogenase gene (*his4*) that prevents it from synthesizing histidine. However, histidinol dehydrogenase gene was integrated into the genome of the recombinant yeast in the process of construction of recombinant protein, resulting in mutation defect complement, which was considered to be an effective way of screening phenotypes (Cregg *et al*. 2000). Because the histidine producing ability of the recombinant *P. pastoris* was not as good as the wild type, it would be a bottleneck of the heterologous expression. Extra histidine needs to be supplied to relieve the pressure of lack of histidine when the expressed heterologous protein was rich in histidine. To study the effect of histidine concentration on xylanase expression, different histidine concentrations of 0.05%, 0.1%, 0.3%, and 0.5% (v/v) were added to BMGY medium with no histidine added as a control. The result is shown in Fig. 2d. With increasing histidine concentration, the xylanase activity was enhanced. The histidine concentration of 0.3% led to the highest activity with 1538 U/mL. When the histidine concentration increased to 0.5%, protein yields began to decrease, which was the result of the metabolism of the recombinant being feedback-restrained by the high histidine concentration.

To further improve the xylanase activity, the transformant with the highest activity was subjected to high-cell-density fermentation in a 5-L fermenter. The end of the glycerol batch phase was indicated by a spike in the DO caused by the exhaustion of glycerol. The cell-wet weight reached about 52 g/L, corresponding to an OD_{600} value of around 35 in the phase. During the glycerol-fed-batch, the biomass increased exponentially to 277 g/L with an OD_{600} value of around 242 when all the glycerol had been consumed and the dissolved oxygen level approached 100% of saturation. After 2 hours, the induction phase began with methanol added at a gradually varying flow until the rate of 10.9 mL/h/L fermentation volume was reached, which lasted about 72 hours. The xylanase expression level of codons optimized gene was increased to 13-fold higher than that of the shake flask culture with the maximum activity of 20424.2 U/mL and a total protein of 584.4 mg/L. The cell wet weight increased up to 310 g/L, and the OD_{600} value reached 320 at the end of the induction phase (Fig. 3).

SDS-PAGE analysis of the fermentation supernatant sample taken after a different induction time point was performed. The recombinant xylanase showed three apparent molecular sizes of about 21, 22.5, and 24 kDa (Fig. 4), which was the same as the native recombinant xylanase. The different bands appearing on the electrophoresis are due to a difference in the degree of glycosylation of the polypeptide chains (Deng *et al*. 2006).

Biochemical Characterization of the Recombinant Syn-xylanase

The biochemical properties of a xylanase will impact its commercial effectiveness. Effects of temperature on activity and stability of the recombinant enzyme are shown in Fig. 5. The optimal temperature was 50°C. The enzyme displayed about 90% of its peak activity in the temperature range 37 to 40°C, *i.e.* the body temperature of animals that might receive the enzyme in their diet. However, enzyme activity declined rapidly at temperatures in excess of 50°C, indicating potential problems if the enzyme was used at high temperatures, such as those used for pelleting or extruding diets (Lawrence 1970).

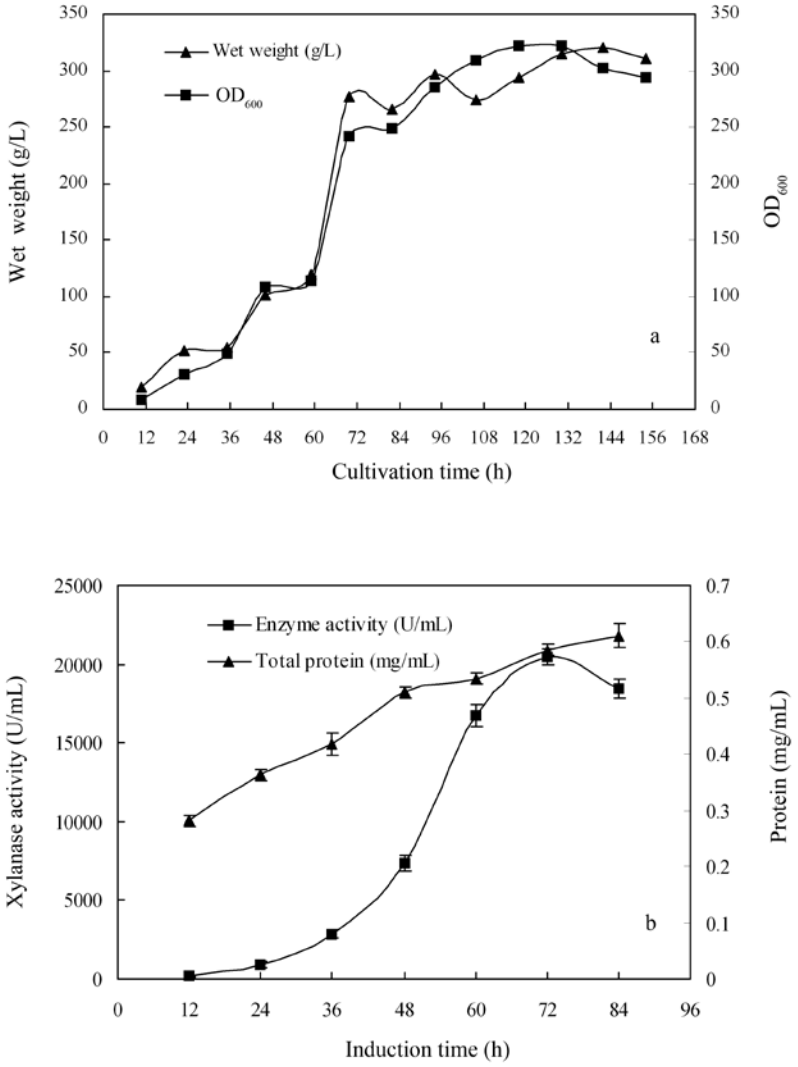

Fig. 3. Growth process and secretion curve of recombinant syn-xylanase in high-cell-density fermentation: (a) Growth process of the recombinant yeast cells (b) Expression of syn-xylanase and secretion of total protein in a 5-L fermenter

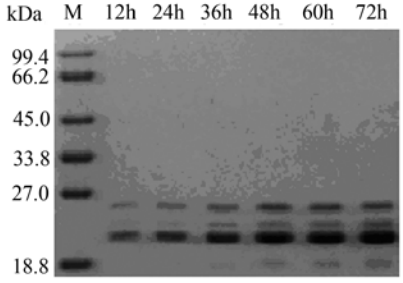

Fig. 4. SDS-PAGE analysis of recombinant syn-xylanase in *P. pastoris*: The fermentation supernatant was collected at different induction times. Lane M: Protein molecular weight standards. The lane heading 12 h, 24 h, 36 h, 48 h, 60 h, and 72 h indicated the induction time.

The optimal pH for the recombinant xylanase was about 5.0. The enzyme retained about 90% of its activity after being incubated at pH 3.0 to 8.0 for 1 hour at room temperature. Enzymes used in animal feeds must survive transit through the stomach, and the pH in the animal's stomach on full feed rarely rises above pH 3.0 (Lawrence 1970).

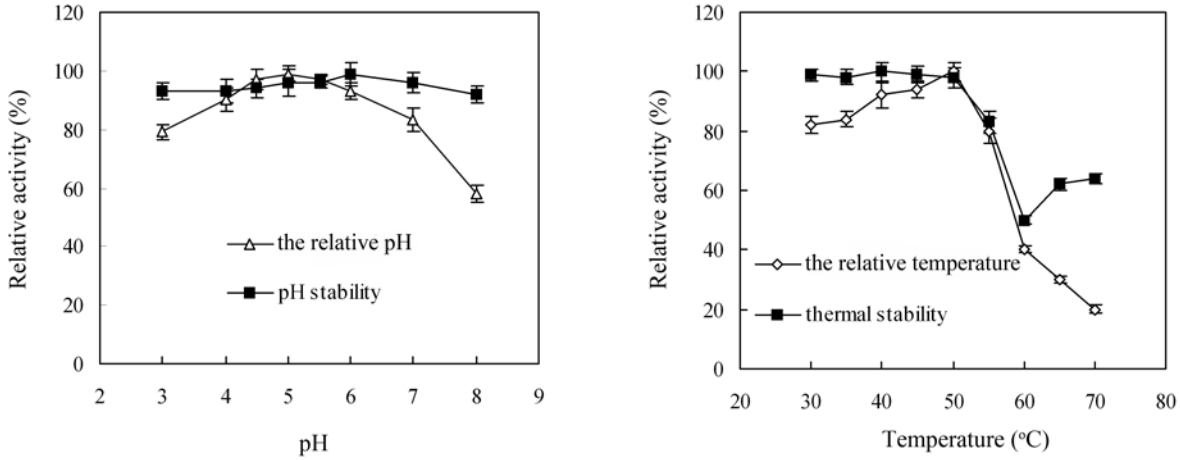

Fig. 5. Characterization of the recombinant syn-xylanase: (a) Effect of pH on xylanase activity. The enzyme activity was determined under different pH values at 50°C. The highest xylanase activity was taken as 100% in an assay of pH optimum. (b) Effect of temperature on xylanase activity. The highest activity was taken as 100% in an assay of temperature optimum. All these assays were performed as described above using 1% birchwood xylan as the substrate.

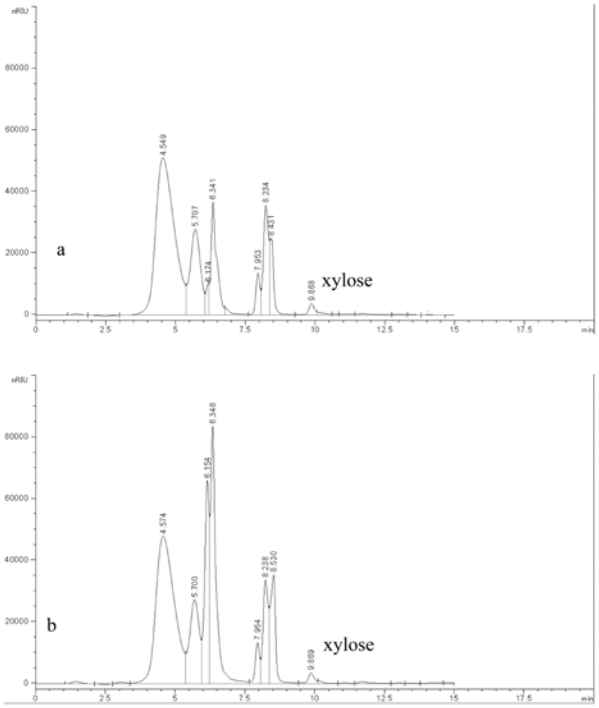

Fig. 6. HPLC analysis of the hydrolysis product: The birchwood xylan was incubated in pH 5.0, 50 mM sodium citrate buffer with syn-xylanase at 45°C for 8 hours (a) and 24 hours (b) 9.869 min implied the retention time of standard xylose.

The kinetic parameters k_m and V_{max} of recombinant xylanase for birch xylan were 16.7 mg/mL and 188.7 mg/mL min, respectively. The enzyme activity was not or only a little affected by 1 mM Fe^{3+}, Ca^{2+}, K^+, Mg^{2+}, Zn^{2+}, Ba^{2+}, Co^{2+}, Al^{3+}, and Fe^{2+}, in which the enzyme was activated by Cu^{2+} and inhibited by Mn^{2+}. The addition of excess EDTA (10 mM) did not affect the activity, suggesting the cation cofactors were not required for the enzymatic reaction.

The hydrolysis product of birchwood xylan by syn-xylanase was analyzed by HPLC, as shown in Fig. 6. As the reaction time increased, birchwood xylan was degraded into xylooligosaccharide and xylose. Xylooligosaccharide was the major hydrolytic product relative to the xylose. After 24 hours of incubation, the yield of xylooligosaccharide increased, but the concentration of xylose remained constant at 0.388 mg/mL. The results showed that the syn-xylanase has great potential in the bioconversion of lignocellulosic waste to xylooligosaccharide. Xylooligosaccharides have attracted more and more interest because of their beneficial effects as bifidobacterium growth-promoting factors (Vasquez *et al*. 2000).

CONCLUSIONS

The *xynB* gene from *A. niger* was optimized, synthesized, and expressed successfully in *P. pastoris* GS115, as shown in this paper. The culture and induction conditions for the recombinant expression of xylanase were optimized. The xylanase activity (20424.2 U/mL) was significantly enhanced by high-cell-density fermentation in a 5-L fermenter; the activity was about 13-fold and 39-fold higher than that of the *syn-xynB* and initial recombinant strain in a shake flask, respectively. Compared to the non-optimized control, the enzymatic properties were not changed. The enzyme showed great potential in the natural biodegradation process of hemicellulose. The present results will also promote the industrial application of the enzymatic process.

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