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Improvement of *Aspergillus sulphureus* Endo-β-1,4-Xylanase Expression in *Pichia pastoris* by Codon Optimization and Analysis of the Enzymic Characterization

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Abstract The gene xynB from *Aspergillus sulphureus* encoding the endo-β-1,4-xylanase was de novo synthesized by splicing overlap extension polymerase chain reaction according to *Pichia pastoris* protein's codon bias. The synthetic DNA and wild-type DNA were placed under the control of a glyceraldehyde-3-phosphate dehydrogenase gene promoter (GAP) in the constitutive expression vector plasmid pGAPzαA and electrotransformed into the *P. pastoris* X-33 strain, respectively. The transformants screened by Zeocin were able to constitutively secrete the xylanase in YPD liquid medium. The maximum yield of the recombinant xylanase produced by the synthetic DNA was 105 U ml^{-1}, which was about 5-fold higher than that by wild-type DNA under the flask culture at 28 °C for 3 days. The enzyme showed optimal activity at 50 °C and pH 5.0. The residual activity remained above 90% after the recombinant xylanase was pretreated in Na_2HPO_4–citric acid buffer (pH 2.4) for 2 h. The xylanase activity was significantly improved by Zn^{2+}. These biochemical characteristics suggest that the recombinant xylanase has a prospective application in feed industry as an additive.

Keywords Endo-β-1,4-xylanase · *Aspergillus sulphureus* · De novo synthesis · Codon optimization · *Pichia pastoris*

Introduction

Xylan, the major constituent of hemicellulose, is a heterogeneous polysaccharide consisting of a β-1,4-linked D-xylose backbone [1]. These compounds are present in the cell wall and in the middle lamella of plant cells [2]. It can be degraded into xylo-oligosaccharides of various lengths by a set of enzymes among which endo-1,4-xylanases (EC 3.2.1.8) are crucial. Xylanases, which are mainly produced by bacteria and fungi, has been well-studied, including their biochemical properties and physiological roles [3]. According to the amino

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acid sequence homology, protein molecular weight, and hydrophobic cluster analysis, xylanases have been classified into two families, named F/10 and G/11 [4–7].

Xylanases are widely used in bread making, clarification of beer and juices, waste treated, and as a biological bleacher in the pulp and paper industry to preserve the environment [2, 8, 9]. Xylanases are also extensively used in animal feed in an attempt to improve feed digestion and the performance of poultry and pigs [10–13]. Recently, much research about xylanase has been focused on alkaline xynlanase for the use in pulp industry [2, 14]. In studies on acid xylanase, researchers are especially interested in exploiting new kinds of wild-type xylanases with potential properties from various microorganisms to extract new genes and obtain good thermostability or other properties [15, 16]. Up to now, many kinds of xylanases have been cloned and expressed in heterologous hosts for potential applications in the food and feed industry [17, 18]. As we know, only a few studies work at the modification of xylanases genes, such as gene synthesis or codon optimization [19].

In previous studies from our laboratory, *Aspergillus sulphureus* CGMCC0608 isolated from soil has been demonstrated to produce β-xylanase in solid medium [20]. The *A. sulphureus* xylanase gene xynB composed of 678 nucleotides (nts) was cloned and expressed in *Escherichia coli* BL21 strain with isopropylthiogalactoside induction [21]. The produced protein formed inclusion body, from which biologically active proteins can only be recovered by complicated and costly denaturation and refolding processes. Proteins expressed in *E. coli* lack eukaryotic posttranslational modifications and are often insoluble and trapped in inclusion bodies. However, the yeast *Pichia pastoris*, a methylotropic single-cell yeast strain, as an eukaryotic expression system, has been developed for expressing heterologous proteins due to its many advantages, such as protein processing, protein folding, and posttranslational modification [22, 23].

In this paper, we present xylanase gene xynB from *A. sulphureus* de novo synthesized by splicing overlap extension polymerase chain reaction (SOE-PCR) and constitutively expressed in *P. pastoris* X-33. The properties of the recombinant enzyme are described in detail.

Materials and Methods

Strains, Vectors, and Chemicals

P. pastoris X-33 and pGAPzαA were purchased from Invitrogen (San Diego, CA, USA). *E. coli* Top10 was used as the host strain. pUCm-T was purchased from Sangon (Shanghai, China). Yeast extract and Tryptone were purchased from OXOID (Hampshire, England). All other chemicals used in this study were of analytical grade commercially available.

Xylanase Gene De Novo Synthesis and Expression Vector Construction

According to the amino acid sequence (GenBank accession number: AAZ95432) of xylanase B obtained from *A. sulphureus*, eight pairs of primers were designed (Table 1). SOE-PCR was performed with these primers using PCR Mix (Tiangen, Beijing, China). At first, primers B-F-1 and B-R-1 were self-complemented through 30 cycles of denaturizing (94 °C for 30 s), annealing (55 °C for 30 s), and extension (72 °C for 1 min). Then, the produced dimer was used as template for the next PCR amplification with primers B-F-2 and B-R-2 under the same conditions. The next cycle of PCR was carried out to obtain

Table 1 Primers used for xynB-opt synthesized.

Primer	Sequence
B-F-1	5-AACGGTTACTTGTCCGTCTACGGTTGGACC ACTGACCCATTGATCGAGTACTACATCGT-3
B-R-1	5-GTACCACCGGAACCTGGGTTGTAGTCGCCGT AGGACTCGACGATGTAGTACTCGATCAA-3
B-F-2	5-TCAAGACATCACCTACTCCGGTACCTTCA CCCCATCCGGTAACGGTTACTTGTCCGTCT-3
B-R-2	5-GTCGTAGACGGAACCGTCGGAAGTGACG GTACCCTTGTAAGTACCACCGGAACCTGGT-3
B-F-3	5-GTAACTTCGTTGGTGGTAAGGGTTGGAAC CCAGGTTCTGCTCAAGACATCACCTACTCC-3
B-R-3	CTTGGATAGAAGCAGCGTTGGTTCTGGTAG CGGTGTAGATGTCGTAGACGGAACCGTCG-3
B-F-4	5-GGTGACGCTGGTTCCTACACCGTCGAGTG GTCCAACGTTGGTAACTTCGTTGGTGGTAA-3
B-R-4	5-TTTTGTCTAACGGACCAGTATTGGGTGAAG GTAGCGGTACCTTGGATAGAAGCAGCGTT-3
B-F-5	5-CTTCTGGACCGACGGTGGTGGTGACGTCA CCTACACCAACGGTGACGCTGGTTCCTACA-3
B-R-5	5-GAAGTGGTTGGAAGTGGTAACAGTACCAC CAACTCTCTTGTTTTGTCTAACGGACCAGT-3
B-F-6	5-CCCCATCCTCTACCGGTGAGAACAACGGT TTCTACTACTCCTTCTGCACCGACGGTGGT-3
B-R-6	5-TGTGAGTACCCAAGTTCATACCCAACTTAG CCCAAGCGTTGAAGTGGTTGGAAGTGGTA-3
B-F-7	5-GAGAGATCTGACGCTTTGCACAAGTTGTC TGAGAGATCTACCCCATCCTCTACCGGTGA-3
B-R-7	5-CCAGAGGATTGGTAACCCTCGGTAGCGA CGATTTGGTAGTTGTGAGTACCCAAGTTCAT-3
B-F-8	5-AGGGAATTCGTTCCACACGACTCTGT CGTCGAGAGATCTGACGCTTTGC-3
B-R-8	5-TTTGCGGCCGCTCATTGAACAGTGATGGA GGAAGAACCAGAGGATTGGTAACCCT-3

B-F-1 and B-R-1, B-F-2 and B-R-2, B-F-3 and B-R-3, B-F-4 and B-R-4, B-F-5 and B-R-5, B-F-6 and B-R-6, B-F-7 and B-R-7, and B-F-8 and B-R-8 were paired. *Underlined parts* in B-F-8 and B-R-8 were introduced *Eco*RI and *Not* I sites

longer DNA sequences. Finally, 644 nts (containing 20 nts of two terminal restriction sites) of DNA fragment amplified with primers B-F-8 and B-R-8, and purified with EZNA Cycle-Pure Kit (Omega, USA), was ligated into pUCm-T vector, and the resulting plasmid was transformed into *E. coli* Top10. The resulting plasmid pUC-B-opt was sequenced using a model 377 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). The synthesized DNA sequence, named xynB-opt, was analyzed by DNAMAN software (Lynnon Biosoft, version 5.2.2, Quebec, Canada).

pUC-B-wt was a plasmid containing wild-type xynB without signal peptide sequence [21]. To construct the *P. pastoris* expression vector, pUC-B-wt and pUC-B-opt were digested with *Eco*RI and *Not* I, and the 624 nts of DNA fragment were recovered through 1% agarose gel, respectively. The purified DNA was ligated into pGAPzαA after pretreatment with *Eco*RI and *Not* I. After being transformed into *E. coli* Top10, the positive clone pGAP-B-wt and pGAP-

B-opt were selected on low-salt LB plates (5 g l^{-1} NaCl, 10 g l^{-1} tryptone, 5 g l^{-1} yeast extract, and 15 g l^{-1} agar) containing 25 μg ml^{-1} Zeocin (Invitrogen, San Diego, CA, USA). The plasmids were confirmed by DNA sequencing using dideoxy chain-termination method [24].

Transformation of *P. pastoris* and Secreting Expression

Linearized recombinant plasmids pGAP-B-wt and pGAP-B-opt with *Bsp*HI were transformed into *P. pastoris* X-33 by a Gene Pulser Xcell™ Electroporation System (Bio-Rad, Hercules, CA, USA) working at 2,000 V and 5 ms. The transformants were screened on YPDS plate (10 g l^{-1} yeast extract, 20 g l^{-1} peptone, 20 g l^{-1} dextrose, 1 mol l^{-1} sorbitol, and 20 g l^{-1} agar) containing 100 μg ml^{-1} Zeocin 3–4 days.

The transformed single colony was inoculated in 10 ml YPD (10 g l^{-1} yeast extract, 20 g l^{-1} peptone, 20 g l^{-1} dextrose) medium and cultivated overnight at 28 °C. The cell culture was inoculated in 20 ml YPD medium by 1:100 dilutions and grown in a 250-ml flask and shaking (250 rpm) at 28 °C for 3 days. Proteins in the supernatant of the culture were separated by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie Brilliant Blue R-250.

The supernatant was dialyzed against to Na_2HPO_4–citric acid buffer (pH 2.4) using Snakeskin™ pleated dialysis tubing (Pierce, Rockford, IL, USA) at 4 °C for 24 h. Protein concentrations of the dialyzed supernatant were determined with Micro-BCA Protein Assay Reagent (Pierce, Rockford, IL, USA) according to the manufacture's instruction. Measurement of the absorbance at 562 nm was conducted with the TECAN-SUNRISE enzyme mark apparatus (SUNRISE, Mannedorf, Switzerland). The supernatant of fermented *P. pastoris* X-33 without exogenetic transformation was used as negative control.

Xylanase Activity Assay

Xylanase activity of xynB was assayed with oat spelt xylan (Sigma X0627, St. Louis, MO, USA) as a substrate, as described as the method of Lu et al. [20]. Different pH values of Na_2HPO_4–citric acid buffers were used to dissolve xylan and to dilute enzyme; 2-ml basic reaction mixture contained 1 ml of diluted enzyme solution and 1 ml of 1.0% (*w*/*v*) oat spelt xylan in Na_2HPO_4–citric acid buffer (pH 4.4). The reaction was incubated at 50 °C for 20 min and the reducing sugar (xylose) was measured by the dinitrosalicylic acid (DNS) according to the standard method [25] and xylose was used as a standard. One unit of xylanase activity was defined as the amount of enzyme that liberated 1 μmol reducing sugar (xylose) from the substrate solution per minute under the assay conditions.

Characterization of Xylanase

Enzyme assays were performed in Na_2HPO_4–citric acid buffer (pH 5.0) at 50 °C. Substrate concentrations were in the range of 0.1–1% (*w*/*v*). The enzyme concentration was 0.78 mg ml^{-1}. The values of the Michaelis–Menten constant (K_m) and maximal velocity (V_{max}) were determined by Lineweaver–Burk plot. The kinetic parameters, turnover number (K_{cat}), and catalytic efficiency value (K_{cat}/K_m) were calculated from initial velocities using concentrations of 1% (*w*/*v*) of oat spelt xylan.

To determine the optimal pH values of the xylanase, the enzyme and xylan substrate were pretreated with Na_2HPO_4–citric acid buffers to make the pH value of reaction mixture

ranging from 2.4 to 7.4. The relative activities of the xylanase were determined by incubating at 50 °C for 20 min.

To investigate the acidophilic stability of the recombinant enzyme, the supernatant of fermentation broth was incubated in the Na_2HPO_4–citric acid buffer of pH 2.4 for 0, 30, 60, 90, and 120 min at room temperature. The standard activity assay was conducted at pH 5.0 and 50 °C for 20 min.

The temperature profile of the recombinant enzyme was determined at 20, 30, 40, 50, 60, and 70 °C. The relative activities of the xylanase were conducted at pH 5.0 for 20 min, using Na_2HPO_4–citric acid buffer.

The thermal stability of the recombinant enzyme was measured under the following conditions: xylanase was incubated for 30 min at the temperature ranging from 20 to 70 °C, respectively. The residual xylanase activity assay was conducted at pH 5.0 and 50 °C for 20 min.

Effects of various metal ions and chemicals on the activity of recombinant xynB were tested at pH 5.0 and 50 °C for 20 min with targeted metal ions and chemical agents at the final concentrations of 10 mM. The reaction mixture without the metal ions or chemicals was used as control.

To determine the specificity of the recombinant xylanase, locust bean gum (Sigma, G0753), barley β-glucan (Sigma, G6513), and lichenan (Sigma, L6133) with concentration of 1 mg ml^{-1} was used as substrates, respectively. Enzyme assays were performed in Na_2HPO_4–citric acid buffer (pH 5.0) at 50 °C.

Results and Discussion

Sequence Optimization and De Novo Synthesis of XynB from *A. sulphureus*

P. pastoris has been routinely used as a heterologous expression system because of its efficient secretion, high expression level, proper protein folding, and very high cell density [22, 23]. However, the bias of codon usage in the foreign gene is beneficial to its high-level expression. Optimal codons of *P. pastoris* have been confirmed according to the synonymous codons used in 28 open reading frames [26]. We hereby presented the *A. sulphureus* CGMCC0608 xylanase gene xynB expression in *P. pastoris* by wild-type gene and de novo synthetic optimal DNA, respectively.

According to the codon bias of *P. pastoris*, *A. sulphureus* xylanase gene xynB (GenBank accession number: DQ168666), without signal peptide sequence, was optimal designed. Eighty-three of nucleotides (nts) in the sequence were changed. The optimal sequence has 52.1% of GC content and shared 86.4% similarity to the wild-type xynB (Fig. 1). The resulting SOE-PCR product, xynB-opt, from agarose gel analysis is shown in Fig. 2. Sequence analysis showed that the DNA was composed of 624 nts and encoded for a protein of 22.2 kDa molecular mass. The amino acid sequence encoded by the synthetic DNA did not show any differences when compared to the wild-type mature xylanase (data not shown).

Constitutive Expression of xynB-wt and xynB-opt in *P. pastoris*

The wild-type xynB and the synthetic xynB-opt were digested with *Eco*RI/*Not* I and ligated into a constitutive expression vector pGAPzαA of *P. pastoris*, in which the foreign protein was secreted as directed by α-mating factor of *Saccharomyces cerevisiae*. The recombinant plasmids were linearized by *Bsp*HI and then electroporated into *P. pastoris* X-33 to

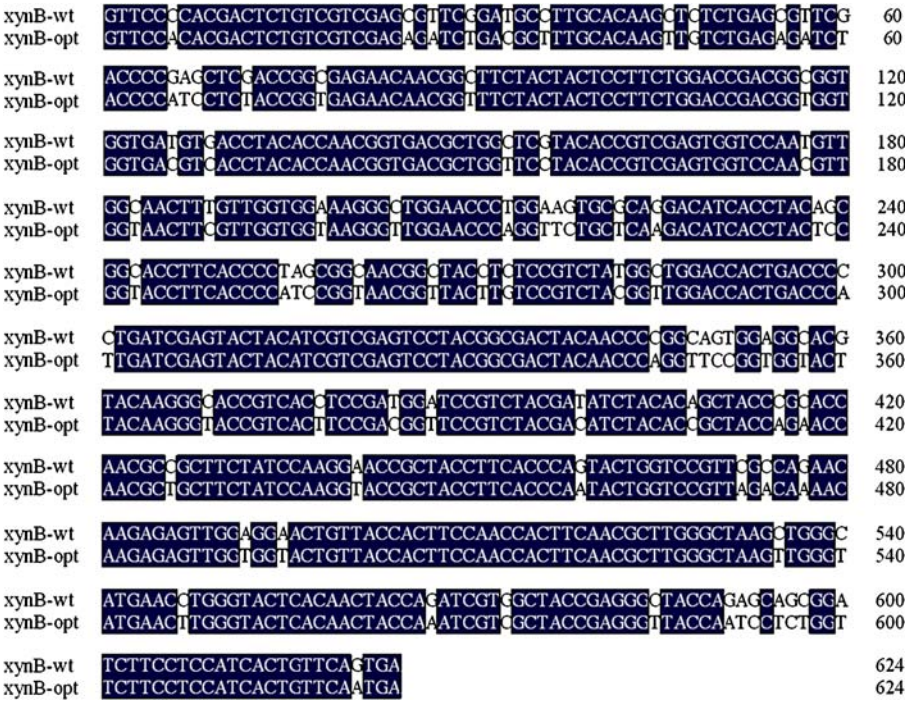

Fig. 1 Alignment of *A. sulphureus* wild-type xynB nucleotide sequence (xynB-wt) with optimized xynB (xynB-opt) generated by DNAMAN (Lynnon Biosoft, version 5.2.2). Identical residues are marked in *black background*

construct a constitutive expression system where the recombinant xylanase was expressed under the control of the GAP promoter.

The transformant screened through 100 μg ml^{-1} of Zeocin selection was cultured in 20 ml of YPD liquid medium and shaking at 28 °C. The supernatant of the culture was analyzed by SDS-PAGE (Fig. 3). A single protein band was detected at a molecular weight of proximately 22 kDa.

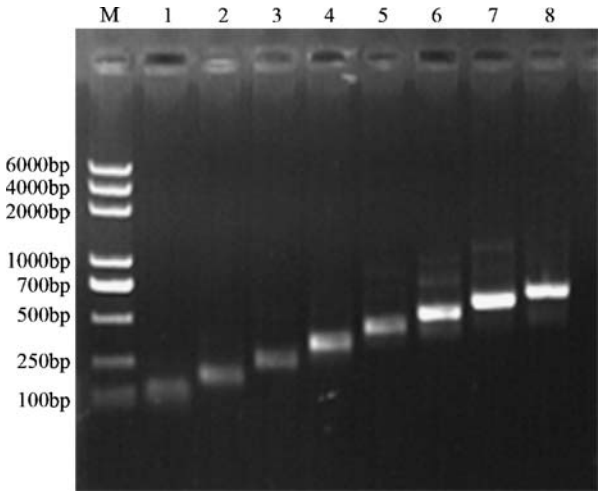

Fig. 2 Results of SOE-PCR detected by 1% agarose gel. *Lane M* molecular weight marker; *lane 1*: dimer amplified with primers B-F-1 and B-R-1. *Lanes 2 to 8* Amplification with primers B-F-2/B-R-2, B-F-3/B-R-3, B-F-4/B-R-4, B-F-5/B-R-5, B-F-6/B-R-6, B-F-7/B-R-7, and B-F-8/B-R-8, respectively

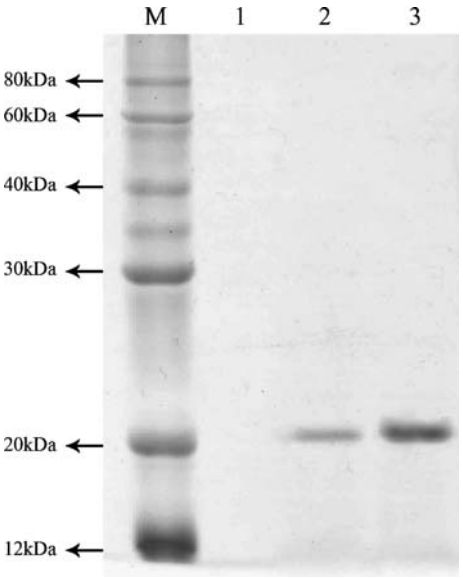

Fig. 3 SDS-PAGE analysis of the recombinant xylanase from *P. pastoris* transformants. *Lane M* protein molecular marker; *lane 1* supernatant of *P. pastoris* X-33; *lane 2* supernatant of X-33/xynB-wt; *lane 3* supernatant of X-33/xynB-opt

As an excellent system for expressing heterologous proteins in secreted forms, *P. pastoris* has perfect protein processing mechanism such as signal peptide cleavage, protein folding, and posttranslational modifications inside the cell and also has favorable capability of heterologous proteins secretion into medium with normal function [27]. Without any purification of the crude enzyme, the pure single protein band was observed in SDS-PAGE, which maybe attribute to the advantages of *P. pastoris* expression system.

Codon optimization is considered as a promising technique for increasing protein expression level [28]. In this experiment, protein concentration of the dialyzed supernatant was determined with a yield of 0.16 mg ml^{-1} in strain X-33/xynB-wt, while a yield of 0.78 mg ml^{-1} was observed in X-33/xynB-opt. The recombinant xylanase in the supernatant of X-33/xynB-wt was detected at 50 °C and pH 5.0 for 20 min, and the catalytic activity reached 24 U ml^{-1}. However, the xylanase activity produced by recombinant strain X-33/xynB-opt reached 105 U ml^{-1}. The control strain, *P. pastoris* X-33 had no detectable xylanase activity. Compared to those of wild-type gene product, the protein yield and enzyme activity by the optimal DNA were significantly improved to nearly 5-folded, which might be potential for further production.

Two mainly heterologous expression systems, directed by inducible promoter AOX1 and constitutive promoter GAP, respectively, were most used in *P. pastoris*. However, in the former one, protein production must be induced with methanol, and the fermentation course must be carried out over a long period. The latter one was highlighted for the foreign gene under the control of the GAP gene promoter that could be efficiently constitutively expressed [19, 29, 30].

Properties of the Recombinant Xylanase

The K_m value of xylanase was 11.14 mg ml^{-1}, and the maximal velocity was 232.56 mol l^{-1} s^{-1} when oat spelt xylan was used as the substrate. The turnover number and the catalytic efficiency value were 6,550.99 s^{-1} and 588.067 ml s^{-1} mg^{-1}, respectively, according to the K_m and V_{max}.

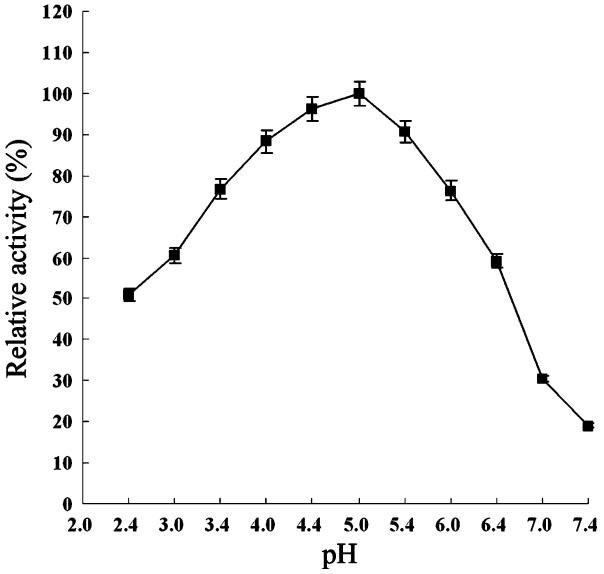

Fig. 4 Optimal pH of the recombinant xylanase

As shown in Figs. 4 and 5, the optimal pH value of the xylanase was 5.0, and acidic stability was similar with the normal distribution curve. The xylanase activity maintained a high level within the range of pH 2.4 to 6.4. Under conditions of pH value more than 6.4, the catalytic activity of enzyme decreased rapidly. In the acidic conditions, from pH 2.4 to 6.4, more than 50% activity of the enzyme was observed compared with the activity in optimal pH buffer, and more interestingly, the residual activity remained above 90% after the recombinant xylanase was pretreated in acidophilic buffer (pH 2.4) for 2 h. In a word, the xylanase has an excellent property of acid resistance and its adaptability in acidophilic reaction conditions could just accord with animal digestive tract. Basing on both of two advantages above, it could be served as a good feed additive.

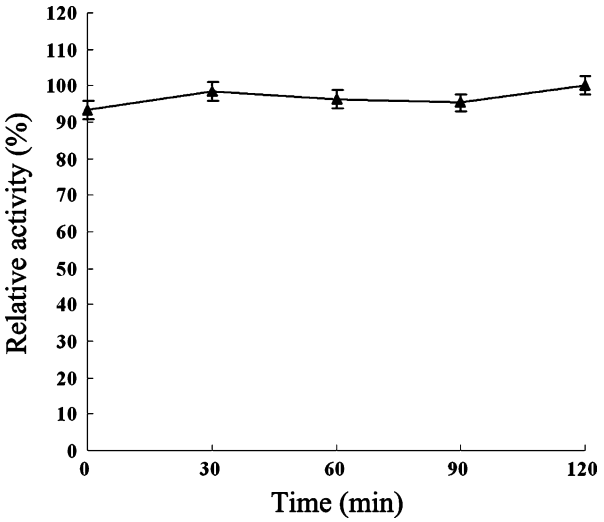

Fig. 5 Acidic stability of the recombinant xylanase in pH 2.4 buffer

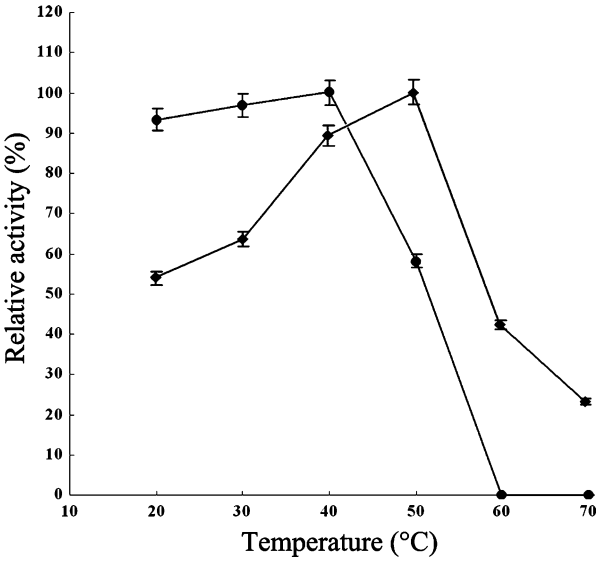

Fig. 6 Effect of temperature on the recombinant xylanase activity. Temperature optimum was determined by measuring the relative activity at pH 5.0; temperature stability was determined by incubating the enzyme at temperatures from 20 to 70 for 30 min, and the enzyme activity was determined at 50 and pH 5.0. *Filled diamonds* temperature optimum; *filled circles* temperature stability

The optimal temperature of the xylanase was 50 °C (Fig. 6). The xylanase activity remained more than 50% compared with the optimal temperature in the range of 20 to 50 °C. However, the activity of the xylanase quickly declined when the temperature was higher than 50 °C. Almost less than 20% activity of the recombinant enzyme was detected at 70 °C. The temperature stability of xylanase is presented in Fig. 6. According to the results, the recombinant protein showed the outstanding stability at the normal temperature below 40 °C. When the xylanase was incubated at 50 °C for 30 min, the residual activity remained about 50% of the maximal enzyme activity. However, no enzymatic activity was observed after heating at higher temperatures (≥60 °C) for 30 min. All of the results illuminated that recombinant xylanase could adapt to the inner temperature of animal, but supplementation of this enzyme in animal feed was not facilitated in high temperatures in the feed processing such as pelletizing or extrusion.

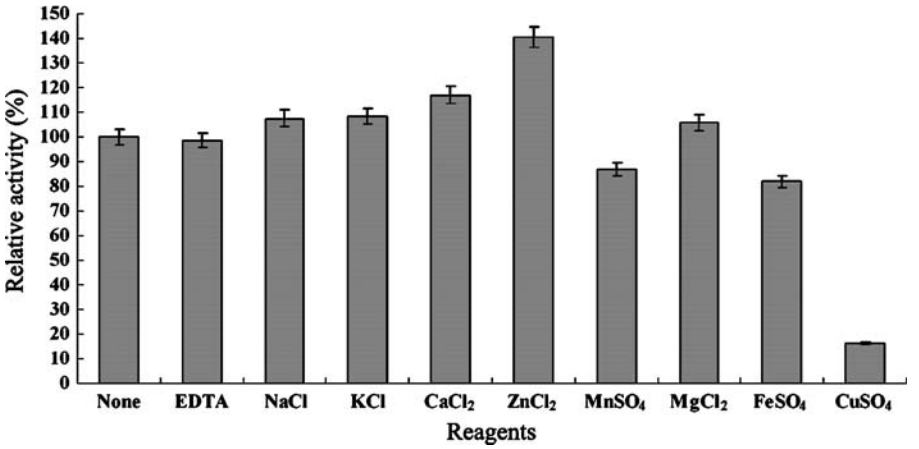

Fig. 7 Effects of chemical reagents on the xylanase

Effects of various metal ions and EDTA of 10 mM on the xylanase activity were detected. As shown in Fig. 7, compared to the activity of the standard xylanase, the enzyme activity was not significantly affected by metal ions, such as EDTA, Na^{+}, K^{+}, Ca^{2+}, Mg^{2+}, and Mn^{2+}. Fe^{2+} and Mn^{2+} inhibited the enzyme activity by 18.0% and 13.2%, respectively. Cu^{2+} was the most inhibitive and the enzyme lost 83.5% of its activity. The value, however, was raised to 140.4% in the presence of Zn^{2+} in the reaction mixtures.

The recombinant xylanase showed no any activity to locust bean gum, barley β-glucan, and lichenan (data not shown).

In this paper, the recombinant protein was efficiently expressed with a yield of 0.78 mg ml^{-1} in the supernatant of fermentation culture. The enzyme displayed optimal activity in oat spelt xylan hydrolysis at 50 °C and pH 5.0. This temperature and pH for optimal enzyme activity of the recombinant xylanase are in the range of some other microorganism that was reported [4]. Another *A. sulphureus* CGMCC0608 xylanase A showed optimum of activity at 50 °C and pH 2.4–3.4 [19]. To our surprise, the metal ion Zn^{2+} has significant improvement to the xylananse activity. At present, this property has not been found in other xylanase. These enzyme characteristics suggest that the recombinant enzyme is suitable for feed additive of animals. Our further study will be focused on pilot scaled-up fermentation of the recombinant *P. pastoris* strain.

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References

1. Singh, S., Madlala, A. M., & Prior, B. A. (2003). *FEMS Microbiology Reviews, 27*, 3–16. doi:10.1016/S0168-6445(03)00018-4.
2. Polizeli, M. L. T. M., Rizzatti, A. C. S., Monti, R., Terenzi, H. F., Jorge, J. A., & Amorim, D. S. (2005). *Applied Microbiology and Biotechnology, 67*, 577–591. doi:10.1007/s00253-005-1904-7.
3. Sunna, A., & Antranikian, G. (1997). *Critical Reviews in Biotechnology, 17*, 39–67. doi:10.3109/07388559709146606.
4. Wong, K. K., Tan, L. U., & Saddler, J. N. (1988). *Microbiological Reviews, 52*, 305–317.
5. Gilkes, N. R., Henrissat, B., Kilburn, D. G., Miller, R. C., Jr., & Warren, R. A. (1991). *Microbiological Reviews, 55*, 303–315.
6. Henrissat, B., & Bairoch, A. (1993). *The Biochemical Journal, 293*, 781–788.
7. Henrissat, B., & Davies, G. (1997). *Current Opinion in Structural Biology, 7*, 637–644. doi:10.1016/S0959-440X(97)80072-3.
8. Beg, Q. K., Kapoor, M., Mahajan, L., & Hoondal, G. S. (2001). *Applied Microbiology and Biotechnology, 56*, 326–328. doi:10.1007/s002530100704.
9. Damaso, M. C. T. (2003). *Applied and Environmental Microbiology, 69*, 6064–6072. doi:10.1128/AEM.69.10.6064-6072.2003.
10. Wu, Y. B., Ravidran, V., Thomas, D. G., Birtles, M. J., & Hendricks, W. H. (2004). *British Poultry Science, 45*, 385–394. doi:10.1080/00071660410001730888.
11. Bedford, M. R., & Classen, H. L. (1992). *The influence of dietary xylanase on intestinal viscosity and molecular weight distribution of carbohydrates in rye-fed broiler chicks* (pp. 361–370). Elsevier: Amsterdam.
12. Barrera, M., Cervantes, M., Sauer, W. C., Araiza, A. B., Torrentera, N., & Cervantes, M. (2004). *Journal of Animal Science, 82*, 1997–2003.
13. Thacker, P. A., & Baas, T. C. (1996). *Animal Feed Science and Technology, 63*, 187–200. doi:10.1016/S0377-8401(96)01028-0.
14. Xia, T., & Wang, Q. (2009). *World Journal of Microbiology & Biotechnology, 25*, 93–100. doi:10.1007/s11274-008-9867-3.

15. Weng, X. Y., & Sun, J. Y. (2005). *Current Microbiology, 51*, 188–192. doi:10.1007/s00284-005-4543-4.
16. Lee, J. W., Park, J. Y., Kwon, M., & Choi, I. G. (2009). *Journal of Bioscience and Bioengineering, 107*, 33–37.
17. Wang, Y. R., Zhang, H. L., He, Y. Z., Luo, H. Y., & Yao, B. (2007). *Aquaculture (Amsterdam, Netherlands), 267*, 328–334. doi:10.1016/j.aquaculture.2007.03.005.
18. Zhou, C. Y., Li, D. F., Wu, M. C., & Wang, W. (2008). *World Journal of Microbiology & Biotechnology, 24*, 1393–1401. doi:10.1007/s11274-007-9622-1.
19. Cao, Y. H., Qiao, J. Y., Li, Y. H., & Lu, W. Q. (2007). *Applied Microbiology and Biotechnology, 76*, 579–585. doi:10.1007/s00253-007-0978-9.
20. Lu, W. Q., Li, D. F., & Wu, Y. B. (2003). *Enzyme and Microbial Technology, 32*, 305–311. doi:10.1016/S0141-0229(02)00292-2.
21. Cao, Y. H., Chen, X. L., He, P. L., & Lu, W. Q. (2006). *Biotechnology Letters, 17*, 858–861.
22. Kim, T. R., Goto, Y., Hirota, N., Dawata, K., Denton, H., Wu, S. Y., et al. (1997). *Protein Engineering, 10*, 1339–1345. doi:10.1093/protein/10.11.1339.
23. Reverter, D., Ventura, S., Villegas, V., Vendrell, J., & Avilés, F. X. (1998). *The Journal of Biological Chemistry, 273*, 3535–3541. doi:10.1074/jbc.273.6.3535.
24. Sanger, F., Nicklen, S., & Coulson, A. R. (1977). *Proceedings of the National Academy of Sciences of the United States of America, 74*, 5463–5467. doi:10.1073/pnas.74.12.5463.
25. Miller, G. L. (1959). *Analytical Chemistry, 31*, 426–428. doi:10.1021/ac60147a030.
26. Zhao, X., Huo, K. K., & Li, Y. Y. (2000). *Chinese Journal of Biotechnology, 16*, 308–311.
27. Sue, M. P., Mariana, L. F., Brian, M., & Linda, M. H. (2005). *Yeast (Chichester, England), 22*, 249–270. doi:10.1002/yea.1208.
28. Nikolay, S. O., Willem, J. S., & Maarten, A. J. (2002). *Protein Expression and Purification, 24*, 18–24. doi:10.1006/prep.2001.1523.
29. Waterham, H. R., Digan, M. E., Koutz, P. J., Lair, S. V., & Cregg, J. M. (1997). *Gene, 186*, 37–44. doi:10.1016/S0378-1119(96)00675-0.
30. Deng, P., Li, D. F., Cao, Y. H., Lu, W. Q., & Wang, C. L. (2006). *Enzyme and Microbial Technology, 39*, 1096–1102. doi:10.1016/j.enzmictec.2006.02.014.