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Cloning of a Gene Encoding Xylanase B from *Aspergillus niger* and its Expression in *Escherichia coli*

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Abstract: In this study, the gene *xynB* encoding xylanase B, obtained from *Aspergillus niger* AN-1 was cloned and efficiently expressed in *E. coli* BL21. The full-length gene contained 658 bp and encoded a mature protein containing 225 amino acids. The purified, recombinant xylanase XYNB showed one band at about 25 kDa. The maximum yeild of the recombinant xylamase was 4.09×10^5 U g^{-1} which was higher than that obtained when *Aspergillus niger* was solid state fermented. The optimal temperature of XYNB was 50°C and recombinant enzyme displayed about 75% of peak activity in the temperature range found in the body of animals to which the enzyme might be fed. The optimum pH of XYNB was about pH 5.0 and the recombinant enzyme retained >80% enzyme activity in the pH range found in the animal gastrointestinal tract where the enzyme might take effect. These enzyme properties suggested that this enzyme could be potentially useful in feed industry.

Key words: Xylanase, *xynB* gene, *Aspergillus niger*, *Escherichia coli*, enzyme

INTRODUCTION

Xylan is one of the major constituents of plant cell walls. As the most abundant hemicellulose, it accounts for >30% of the dry weight of terrestrial plants. Xylan thus belongs to the main food source of domestic animals (Selle *et al*., 2009). Several enzymes are involved in the hydrolysis of xylan polymers of which the most important are the endo-1,4-β-xylanases (EC 3.2.1.8; Kusters van Someren *et al*., 1992). The inclusion of these enzymes in cereal grain based pig and poultry diets has been a routine for nearly 20 years. Their primary purpose is to degrade the non-starch polysaccharides to counteract the negative impact of increased gut viscosity induced by soluble arabinoxylans (Steenfeldt *et al*., 1998; Kim *et al*., 2008).

A. niger xylanase is of particular commercial interest because of its low pH optimum and significant stability in acidic environment which is essential for releasing important nutrients in such acidic environments as animal stomach (Krengel and Dijkstra, 1996). In a previous study conducted in the laboratory, a specific endo-1,4-β-xylanases (EC3.2.1.8) obtained from *A. niger* AN-1, produced by solid-state fermentation was identified as an excellent feed additive for broilers. However, detailed analysis of this enzyme was hampered by the presence of several enzyme activities in the fungal preparation and the inability to obtain the specific xylanolytic enzyme in a pure form. Recombinant DNA procedures allow the design of a production route for the specific xylanase. In subsequence research, a xylanase A gene (*AnxA*) from this fungus had been cloned and expressed in *E. coli* BL21. Expression product of AnxA in *E. coli* BL21 is inclusion body and the xylanase activity is very low.

To achieve a comprehensive understanding of the xylanase system of the *A. niger* AN-1 and get further study on constructing xylanases with excellent enzyme properties that suitable for feed industry, in this research, a xylanase B gene (*AnxB*) was cloned from *A. niger* AN-1 and expressed in *E. coli* BL21. In addition, the pH and thermal stability of the recombinant enzyme was investigated to determine the suitability of the enzyme for use in animal feed.

MATERIALS AND METHODS

Strains and plasmids: In this study, *A. niger* AN-1 was isolated from the soil of Zhejiang University planting base (Hangzhou, China). *E. coli* Top10F' and BL21 strains were used as host cells for genes cloning and expression. The three strain listed above were contained in the laboratory. The cloning Vector pUCm-T was obtained from Sangon (Shanghai, China) and the expression vector pET-28a(+) was the production of Invitrogen (San Diego, CA).

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Reagents: RNA Isolation kit, Fast HiFidelity PCR kit and Agarose Gel Recovery kit were products of TianGen (Beijiang, China). PrimeScript 1st Strand cDNA Synthesis kit, Dnase I, T4 DNA ligase and restrict enzymes NdeI, EcoRI were obtained from Takala (Dalian, China). Oligonucleotides were synthesized by Shangon (Shanghai, China). Ni-NTA agarose resin was from Qiagen (Hilden, Germany). Anti-His antibody and Goat anti Mouse IgG-HRP were achieved from TransGen (Beijiang, China). Birch xylan and dinitrosalicylic acid (DNS) were purchased from Sigma (St. Louis, MO). All other chemicals were of analytical grade.

Cloning of the *xynB* gene from *A. niger* and analysis: The strain *A. niger* AN-1 was activated on Czapek's agar plates (0.3% $NaNO_3$, 0.05% g KCl, 0.1% K_2HPO_4, 0.001% $Fe_2(SO_4)_3$, 0.05% $MgSO_4$ 3% sucrose, 2% agar, pH 7.2) at 30°C for 72 h. Then the spores of the fungus were harvested through flushing the colonies on the plates with stroke-physiological saline solution. Appropriate amount of the spore suspension was inoculated in 200 mL liquid Czapek's medium and incubated at 30°C with constant shaking at 150 rpm for 72 h. The mycelia (0.2 g) were harvested by centrifugation, washed with distilled water. Disruption of mycelia and extraction of RNA were performed with RNA Isolation kit (TianGen, Beijing, China) following the manufacturer's instruction. Then, the cDNA template was synthesized by PrimeScript 1st Strand cDNA Synthesis kit (TaKaRa, Dalian, China) according to the manufacturer's instruction.

A pair of cloning primers was designed based on the 5'terminal and 3'terminal amino acid sequences of xylanases from *A. niger* (AF490982, EU430370, AY536639, AY126481) reported in GenBank (Ohta *et al*., 2001). The forward primer was 5'-ATGCTCACCAAGAACCTT-3' and reverse primer was 5'-TTACTGAACAGTGATGGA-3'. Polymerase Chain Reaction (PCR) using these primers was performed using HiFi Taq polymerase (TaKaRa) under the following conditions: denaturation at 94°C for 5 min and then 35 cycles of denaturation at 94°C for 40 sec, annealing at 53−0.1°C per cycle for 40 sec and extension at 72°C for 50 sec followed by a final extension at 72°C for 8 min. Then, the PCR product was gel eluted using a Agarose Gel Recovery kit (TianGen) then cloned into pUCm-T (Sangon, Shanghai, China) and the resulting plasmid (pUCm-xynB) was then transformed into *E. coli* 10F' through the Calcium Chloride Method. Transformants were screened on LB plates (10 g L^{-1} tryptone, 10 g L^{-1} NaCl, 5 g L^{-1} yeast extract, 15 g L^{-1} agar, pH 7.5) supplemented with 100 μg mL^{-1} ampicillin and 20 μg mL^{-1} X-Gal by convenient blue-white selection and the nucleotide sequence was determined by the Dideoxy Chain Termination Method (Sanger *et al*., 1977). The nucleotide sequence was entered into DNAMAN (Lynnon Biosoft, Version 5.2.2) to estimate the molecular weight, deduce the amino acid sequence and establish phylogenetic relationships between the *A. niger* xylanase and homologous xylanases. The Basic Local Alignment Search tool (Blast Program) was used to search for homologies to other sequences deposed in the NCBI database of xylanases.

Construction of expression plasmid: The cleavage site of the signal peptide was determined by SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/) and the open reading frame of the *xynB* gene was amplified by PCR from the cloning plasmid with the forward primer 5'-CCATATGTCGACCCCGAGCTCGACC-3' containing the NdeI site and reverse primer 5'-GGAATTCCTGAACAGTGATGGAGGA-3' containing the EcoRI site under conditions of denaturation at 94°C for 5 min and then 35 cycles of denaturation at 94°C for 40 sec, annealing at 55−0.1°C per cycle for 40 sec and extension at 72°C for 50 sec followed by a final extension at 72°C for 8 min. Then, the PCR product was gel eluted and cloned into the T vector, the resulting plasmid was transformed into *E. coli* 10F' and screened. The recombinant plasmid was extracted from the positive colonies and the DNA fragment xynB digested by NdeI and EcoRI from the plasmid was ligated into corresponding sits of pET-28a(+) with a polyhistidine (6His) tag formed in the C-terminus. Then, the resulting plasmid pET-28a(+)/xynB was transformed into *E. coli* BL21 and screened on LB plate containing 50 μg mL^{-1} kanamycin.

Expression and purificatiom of recombinant protein: The transformants were incubated in LB medium (containing 50 μg mL^{-1} kanamycin) at 37°C overnight. The 1 mL of the aliquots was transfer-inoculated into 100 mL LB medium (containing 50 μg mL^{-1} kanamycin) and shaken at 37°C for 2-3 h. The recombinant proteins were induced with treatment by final concentration of 1 mmol L^{-1} isopropyl β-D-thiogalactopyranoside (IPTG) for 5-6 h. The recombinant protein was purified with Ni-NTA column (Qiagen, Hilden, Germany) according to the manufacturer's instruction.

SDS-PAGE and Western blot assay: The transforments were lysed by ultrosonic wave and the total proteins of the bacteria were separated by 12% SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) on a Mini-II apparatus (BioRad, USA). The supernatant of *E. coli* BL21 transformed with vector pET-28a(+) lysate was used as a

negative control. The protein concentration of the recombinant enzyme was measured by the Dye-Binding Assay Method of Bradford with the Bovine Serum Albumin (BSA) as the standard (Bradford, 1976). Western blot detection of the expressed proteins was performed according to the method of Sambrook *et al.* (1989). Proteins were transferred to a Polyvinylidene Difluoride (PVDF) membrane (Millipore, USA) using the Mini Trans-Blot® cell (Bio-Rad). The Anti-His antibody (TransGen, Beijiang, China) was used as the primary antibody and Goat anti Mouse IgG-HRP (TransGen, Beijiang, China) as the secondary antibody. The bound peroxidase was detected colorimetrically with a Diaminobenzidine (DAB) Substrate kit (Zhongshan, Beijing, China).

Xylanase activity assay: The xylanase activity was measured with 1% birch wood (w/v) as substrate at 50°C in McIlvaine's buffer (pH 5.0) (Bailey *et al*., 1992). Reducing sugars freed by enzymatic hydrolysis were quantified by the dinitrosalicylic acid (DNS) (Miller *et al*., 1960). One unit of xylanase activity was defined as the amount of enzyme that produced 1 μmol of xylose equivalent per minute.

pH optimum and stability: The effect of pH on xylanase activity was measured over a range of pH 3.0-7.0 (McIlvaine's buffer system) and 8.0-9.0 (0.2 M glycine, 0.2 M NaOH buffer system) at 60°C. The pH stability of the enzyme was determined by incubating the xylanase in various pH buffers at 37°C for 30 min. The residual activity was estimated following the procedure described above.

Temperature optimum and stability: The effect of temperature on the enzyme activity was estimated at optimal pH at temperature ranging from 30-90°C. The thermo stability of xylanase was determined by preincubating the enzyme in the absence of substrate at different temperature for 2 min, respectively then cooling on ice for 5 min before residual xylanase activity measurements.

RESULTS AND DISCUSSION

Cloning and sequence analysis of *xynB* gene from *A. niger* AN-1: The total RNA of *A. niger* AN-1 was obtained to synthesize cDNA. Then primers based on the nucleotide sequences of endo-1,4-β-xylanases from *A. niger* reported in GenBank were employed to amplify the full length cDNA. Nucleotide sequence analysis showed that the 638 bp cDNA fragment is an entire open reading frame (Fig. 1), the deduced protein consisted of an N-terminal signal sequence of 37 amino acids (http://www.cbs.dtu.dk/services/SignalP/) followed by a mature endo-1,4-β-xylanases containing 188 amino acids with a predicted molecular mass of 25 kDa. Homology comparison indicated that *xynB* coding sequence was similar to that of some of the most reported endo-1,4-β-xylanases genes. It was 99.85, 99.71, 99.71 and 98.82% identical to the endo-xylanases from *A. niger* (DQ174549), *A. niger* (AF490982), *A. niger* (AY536639) and *A. niger* (EU430370), respectively. The alignment of these homologous sequences is given in Fig. 2.

Expression, purification and Western-blot assay of the recombinant protein: xynB was then inserted into

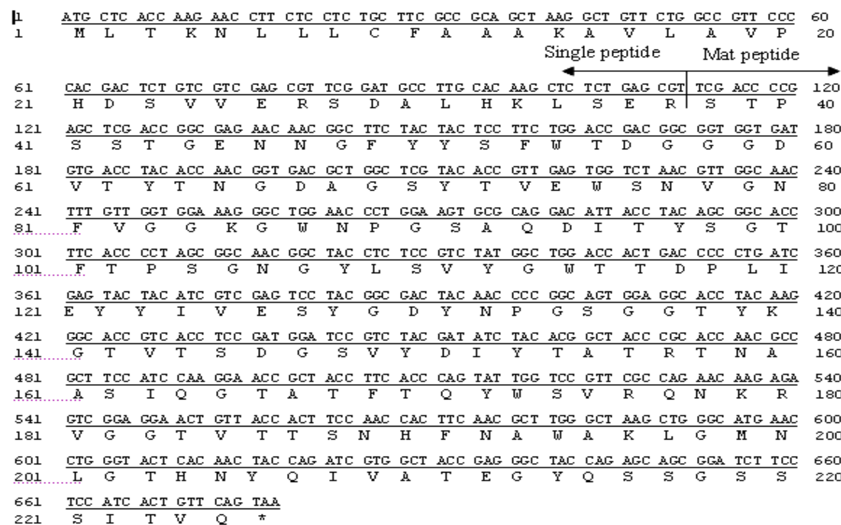

Fig. 1: Nucleotide sequence and deduced amino acid sequence of xylanase gene *xynB* from *Aspergillus niger*

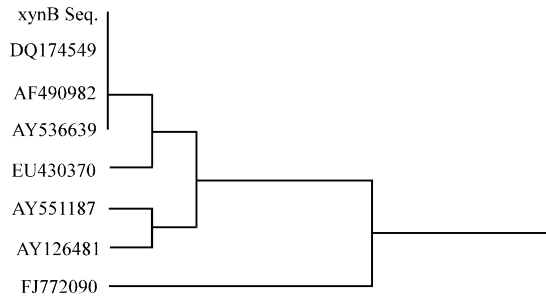

Fig. 2: Graph of genetic distance of xynB from *A. niger*

pET-28a(+), yielding pET-28a (+)/xynB with His-tag in the plasmid and transformed into *E. coli* BL21. The optimized expression of the recombinant protein XYNB was induced with 1 mmol L^{-1} IPTG at 37°C for 6 h. The expressed protein was purified by Ni-NTA metal affinity chromatography from the whole cell lysate. Band of approximately 25 kDa in crude protein extracted from the cell lysate of transformants induced by IPTG and purified protein were revealed on SDS-APGE (Fig. 3a). Western blot analysis indicated that the protein was recognized specifically by Anti-His antibody demonstrating that the expressed heterogenous protein was recombinant xylanase (Fig. 3b). The specific activity of the crude protein extracted from the cell lysate of transformants induced by IPTG and purified protein were 201.44 and 409.23 U mg^{-1}, respectively much higher than that obtained from *A. niger* by solid state fermentation had been reported before (Park *et al*., 2002) while no xylanase activity was detected in the lysate without induction of IPTG under the same culture conditions.

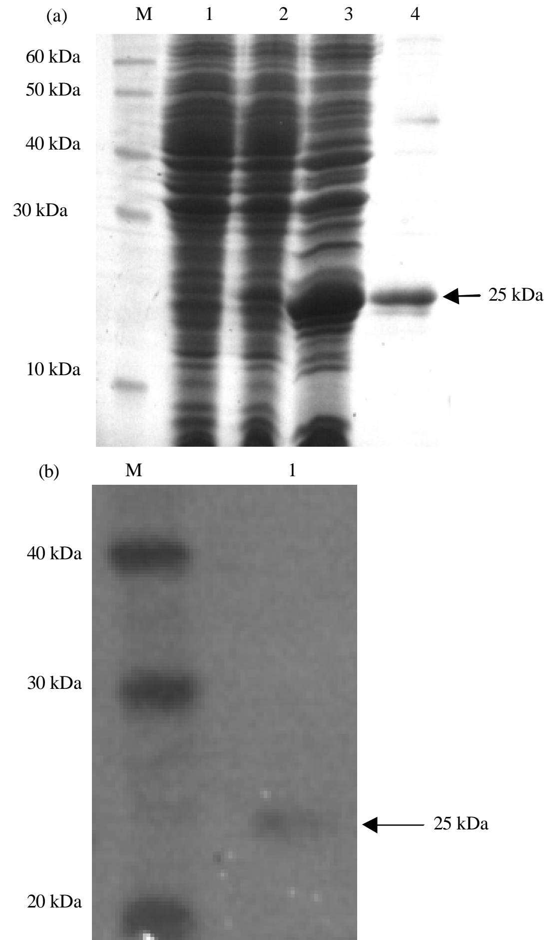

Fig. 3: a) SDS-PAGE analysis of recombinant protein XYNB. Lane M: Protein molecular marker; Lane 1: Total protein of pET-28a(+) transformant; Lane 2: Total protein of pET-28a(+)/xynB induced without IPTG; Lane 3: Total protein of pET-28a(+)/xynB transformant induced by IPTG; Lane 4: Purified XYNB by Ni^{+}-NTA column and b) Western Blot analysis of recombinant protein XYNB. Lane M: Protein molecular marker; Lane 1: Total protein of pET-28a(+)/xynB transformant induced by IPTG

pH optimum and stability of XYNB: The effects of pH on xylanase activity and the stability of the recombinant enzyme XYNB are presented in Fig. 4. The XYNB showed high activity in a pH range of 3.0-6.0 with the optimal pH at 5.0, almost same as that of the xylanase B reported for other fungi. The enzyme retained about 86% of its activity after being incubated at pH 3.0 for 30 min at 37°C. This is important as enzymes used in animal feeds must survive transit through the stomach and the pH in the stomach of animals on full feed rarely rises above pH 3.0 (Kim *et al*., 2006).

Temperature optimum and stability of XYNB: The effects of temperature on xylanase activity and the stability of the recombinant enzyme are presented in Fig. 4b. The XYNB activity increased with the rise of temperature, reached a maximum at 50°C then decreased rapidly with the temperature. The enzyme displayed about 75% of peak activity in the temperature range from 37-41°C which is the body temperature of the animals that might receive the enzyme in their diet. Although, XYNB is stable below 60°C, it lost enzyme activity rapidly above 60°C which indicates potential problems if the enzyme is used in diets processed at high temperatures such as those used for pelleting (Kirkpinar and Basmacioglu, 2006) or extruding (Amornthewaphat *et al*., 2005) diet.

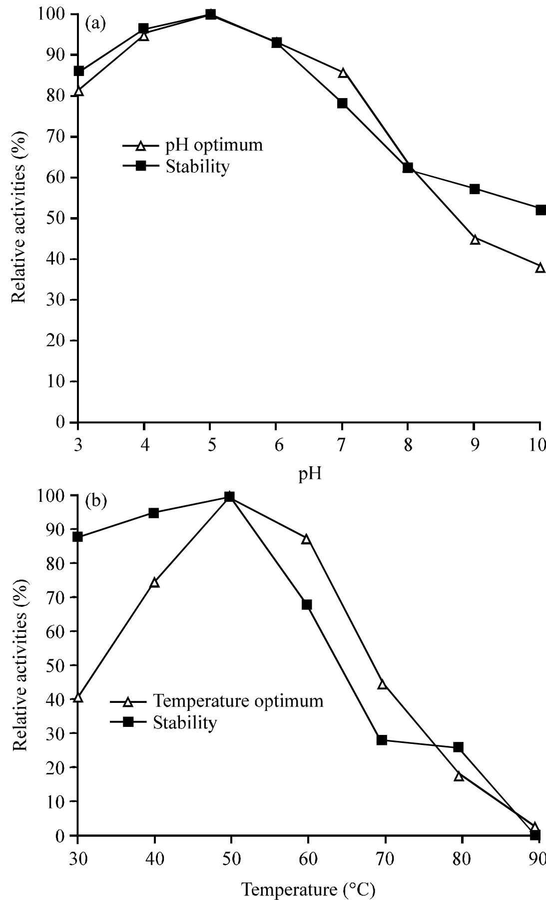

Fig. 4: a) The effects of pH on xylanase activity and b) the effects of temperature on xylanase activity

CONCLUSION

Researchers hereby present cloning a gene encoding xylanase B from *Aspergillus niger* and its expression in *E. coli*. From sequence analysis, it appeared that the recombinant enzyme belonged to family G of glycoside hydrolases. The gene *xynB* cloned from *A. niger* AN-1 was efficiently expressed in the *E. coli* BL21. The recombinant xylanase XYNB was purified and detected by Western-blot assay. The yield of the recombinant xylanase was higher than that obtained from *A. niger* by solid state fermentation which have reported before. XYNB displayed high efficiency in birch wood xylan hydrolysis in the pH range of 3.0-8.0 and at temperatures up to 50°C. These enzyme properties suggest that this enzyme could be used as a feed additive working in the gastrointestinal tract under acidic conditions. Additional studies will be focused on substitution of the *N. terminus* of xynB with the corresponding region of thermal stable xylanase to obtain a hybrid xylanase with excellent thermostability.

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