

*Full Length Research Paper*

# Cloning, sequencing and expression of a novel xylanase cDNA from a newly isolated *Aspergillus awamori* in *Pichia pastoris*

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**A strain SH 2016, capable of producing xylanase, was isolated and identified as *Aspergillus awamori*, based on its physiological and biochemical characteristics as well as its ITS rDNA gene sequence analysis. A xylanase gene of 591 bp was cloned from this newly isolated *A. awamori* and the ORF sequence predicted a protein of 196 amino acids with a molecular mass about 21 kDa. An expression plasmid carrying the gene under the control of the methanol regulated alcohol oxidase gene (AOX1) promotor was introduced into *Pichia pastoris*, and xylanase gene was successfully expressed into the medium using methanol as inducer. Xylanase with 6 his tags was purified using Ni<sup>2+</sup>-NTA column. The characteristics of purified xylanase were investigated.**

**Key words:** *Aspergillus awamori*, cDNA, cloning, xylanase, eukaryotic expression, purification and characterization.

## INTRODUCTION

Xylanases (EC 3.2.1.8) are glycosidases, which can catalyze the endohydrolysis of 1,4-β-D-xylosidic linkages in xylan. Xylanases derived from fungi and bacteria have attracted a great deal of attention in recent years because of their biotechnology potential in various industrial processes (Polizeli et al., 2005). They are produced on an industrial scale for use as bleaching agents in the paper and pulp bleaching industry (Vicuna et al., 1995). Along with glucanase, pectinase, cellulose, proteases, amylases, phytase, lipase and xylanase are also widely used in the degradation of arabinoxylans in feed ingredients, to reduce the viscosity of the raw material and improve nutrition (Silversides et al., 2006). In food industry, they are used as food additives in wheat flour for improving dough handling and quality of baked products (Maat et al., 1992; Jiang et al., 2005). In brewing industry, the addition of exogenous xylanase during the

mashing process was found to be an effective strategy to solve arabinoxylans problems such as high wort viscosity, membrane plugging, and decreased rate of filtration (Lu et al., 2005).

Xylanase production in industrial scale is based on a microbial biosynthesis. Filamentous fungi have demonstrated a great capability for secreting a wide range of xylanases, with the genus *Aspergillus* (Shah and Madamwar, 2005), *Penicillium* (Li et al., 2007a, 2007b) and *Trichoderma* (Azin et al., 2007) being the most extensively studied and reviewed among the xylan-producing fungi. There are two possibilities for cultivation of microbial xylanase producing strain: submerged cultivation and solid-state. Currently 80 - 90% of commercial xylanase are produced in submerged culture because it has a higher degree of processes intensification and a better level of automation (Polizeli et al., 2005). Many xylanase genes were cloned and expressed in *E. coli* or *P. pastoris*. Some recombinant xylanases were characterized and widely used. For its wide applications and remarkable requirement of market, finding new strains capable of producing xylanase or cloning and expressing

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xylanase genes with new properties have become hot issues in xylanase research filed (Hessing et al., 1994; Polizeli et al., 2005).

In this paper, a new gene encoding the xylanase from a newly isolated *A. awamori* has been cloned and expressed in *P. pastoris* X33. Then the optimum temperature and pH, and the thermal stability of the purified xylanase were determined to investigate their potential in the industrial application.

## MATERIALS AND METHODS

### Strains and cultivations

*Aspergillus* sp. SH 2016 was selected from strains maintained locally. The culture medium was composed of  $\text{NH}_4\text{Cl}$ , 9 g/L;  $\text{KH}_2\text{PO}_4$ , 1 g/L;  $\text{NaNO}_3$ , 1 g/L;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 1 g/L;  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.3 g/L; yeast extract, 1 g/L. The initial pH was adjusted to 8.0. Erlenmeyer flasks (250 mL) containing 50 mL medium were inoculated with 1 mL of  $1 \times 10^6$  spores/mL suspension prepared from a fresh PDA slants of *Aspergillus* sp. SH 2016 for incubating at 30 °C on a rotary shaker (150 rpm).

*Escherichia coli* JM109 was used as the host for plasmids pMD18-T-xynA and pPICZaA-xynA transformation. *E. coli* transformants were grown in Luria-Bertani (LB) medium. *Pichia pastoris* X33 was used as a host for expression of pPICZaA-xynA. *P. pastoris* X33 transformants were grown in solid selective YPDS medium (yeast extract (1%, w/v), peptone (2%, w/v), dextrose (D-glucose, 2%, w/v), sorbitol (1 M) and Zeocin (100 µg mL<sup>-1</sup>).

BMGY medium [5 g yeast extract, 10 g peptone, 50 mL 1 M KPO4 buffer pH 6.0, 1.7 g yeast nitrogen base, 5 g ammonium sulfate and 5 mL glycerol were autoclaved at 121 °C for 30 min and cooled down to room temperature, biotin (500 X 1 mL) and histidine (96 X 5.2 mL) were added into medium] was used for cultivation of recombinant *P. pastoris* containing xylanase. BMMY medium [5 g yeast extract, 10 g peptone, 50 mL 1 M KPO4 buffer pH 6.0, 1.7 g yeast nitrogen base and 5 g ammonium sulfate were autoclaved at 121 °C for 30 min, and cooled down to room temperature, methanol (2.4 mL), Biotin (500 X 1 mL) and histidine (96 X 5.2 mL) were added into medium] was used for induction and expression of xylanase in *P. pastoris*.

### Isolation of strains producing xylanase

Various strains including *Aspergillus* sp. SH-2016 were isolated based on their morphology from soil samples, and the detailed procedure of isolation was performed according to the methods described by Abrusci et al. (2005).

### Identification of strain *Aspergillus* sp. SH-2016

The identification of the strain was based on standard morphological characterization (Abrusci et al., 2005), nucleotide sequence analysis of enzymatically amplified ITS rDNA, and the internal transcribed spacer (ITS) region including 5.8 S rDNA. For the morphological characterization of the strain, observations were made with both an optical microscope and a scanning electron microscope. For the sequence analysis, chromosomal DNA was isolated by the methods of Doyle and Doyle (1987). Amplification was carried out with primer set pITS1 (5'-TCCGTAGGTGAACCTGCCG-3') and pITS4 (5'-TCCTCCGCTTATTGATATGC-3') in a thermal cycler (Bio-Rad, USA) under the following conditions: 3 min at 95 °C, 35 cycles of 30

s at 95 °C, 30 s at 53 °C, 2 min at 72 °C and one final step of 10 min at 72 °C. For each PCR reaction 5 µL of PCR products were examined by electrophoresis at 70 V for 2 h in a 1% (w/v) agarose gel in 1×TAE buffer (0.4 M Tris, 50 mM NaOAc, 10 mM EDTA, pH 7.8) and visualized under UV light after staining with ethidium bromide (0.5 µg/mL). The resulting PCR fragment was ligated with pMD18-T (Takara, Japan) by using the T/A cloning procedure (Liu and Sun, 2004). The constructed vector was transferred to the competent cell *E. coli* JM109 according to the method of Chung et al. (1989), and then spread on the LB plate containing the X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside), IPTG (isopropyl-1-thio-β-D-galactoside) and ampicillin (50 µg/mL). Subsequently a positive clone, designated *E. coli* JM109/pMD18-T-ITS, was obtained. ITS DNA was sequenced on both strands with an Applied Biosystems Model 377 Automatic DNA sequencer, and a dye-labeled terminator sequencing kit (Applied Biosystems, Foster, CA, USA). The sequences obtained were compiled and compared with sequences in the GenBank databases using BLAST program. Sequence analysis was performed using SEQBOOT, NEIGHBOR-JION and DNASENSE of Phylips (Version 3.572) softwares and FITCH, DRAWGRAM, and the alignment match was then used to construct the neighbor-joining phylogenetic tree. Evaluation of the MicroSeq database system and comparison to other frequently used systems are from DDBJ/EMBL/GenBank.

### Preparation of total RNA from *Aspergillus* sp. SH-2016

The cells were harvested by filtration, washed twice with phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 4.3 mM  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$  1.4 mM  $\text{KH}_2\text{PO}_4$  pH 7.3), then frozen in liquid N<sub>2</sub>, ground and transferred into a fresh tube containing lysis buffer (6 M guanidinium hydrochloride, 37.5 M citric acid, 0.75 M N-lauroyl sarcosine sodium, 0.15 M β-sulphydryl ethanol), and acid guanidium phenol chloroform method according to Chomczynski and Sacchi (1987) was used to isolate the total RNA from *Aspergillus* sp. SH-2016. Poly(A<sup>+</sup>) RNA was selected from total RNA by using Oligotex-dT30 (Promega, Madison, WI, USA).

### cDNA synthesis and PCR amplification

First strand cDNA was synthesized by RT-PCR with Oligo(dT)15 using mRNA isolated from *Aspergillus* sp. SH-2016 according to manufacturer's protocol (Clontech, Mountain View, CA, USA). The cDNA of *Aspergillus* sp. SH-2016 encoding xylanase was amplified by the 3' race (rapid amplification of cDNA ends) with the primers 5'-GCTCCTGTGCCGAACCTG-3' and Oligo(dT)15 which were designed based on the sequences of NH2-terminus and the cDNA encoding xylanase reported on the National Center for Biotechnology Information (NCBI) and the structure of mRNA respectively, and the first strand cDNAs synthesized above were used as the template. PCR was carried out in 50 µL using a Thermocycler (Bio-Rad, Hercules, CA, USA). PCR products were visualized on a 1.5% ethidium bromide stained gel. The resulting PCR fragment was ligated with pMD18-T (Takara, Otsu, Shiga, Japan) by using the T/A cloning procedure. The constructed vector was transferred to the component cell *E. coli* JM109 according to the method of Chung et al. (1989), and then spread on the LB plate containing the X-gal, IPTG and ampicillin (50 µg mL<sup>-1</sup>). Subsequently a positive clone, designated *E. coli* JM109/pMD18-T-xynA, was obtained. DNA was sequenced on both strands with an Applied Biosystems Model 377 Automatic DNA sequencer, and a dye-labeled terminator sequencing kit (Applied Biosystems, Foster, CA, USA). The sequences obtained were analyzed by software DNAMEN (Version 4.0, Lynnon Biosoft, Canada), then were compiled and compared with sequences in the GenBank databases using the BLAST program.

### Construction of expression vector of cDNA encoding xylanase

ORF fragment of xylanase was obtained using proof-reading Pyrobest DNA polymerase (Takara, Otsu, Shiga, Japan) in the presence of the primers 5'-ATTGAATTCGCACCTGTTCTGAGCCTG-3' (underlined part: EcoRI site) and 5'-GGTCTCGAGTGATGAAATTGTAACGGAG-3' (underlined part: XhoI site), which were designed according to the sequence resulted from pMD18-T-xynA. The PCR products and the vector pPICZaA (Novogen, New Canaan, CT, USA) were digested with EcoRI and XhoI, recovered through agarose gel electrophoresis, and then ligated by T4 DNA ligase. The ligated plasmid pPICZaA-xynA was transformed into *E. coli* JM109. A single colony of the transformant was selected and transferred into 3 ml LB broth with 100 µg ml<sup>-1</sup> ampicillin and incubated overnight with vigorous shaking (200 rpm) at 37°C. The culture was used to extract recombinant plasmid through alkaline lysis procedure (Sambrook et al., 2001). The plasmid was digested with EcoRI and XhoI in order to identify if the recombinant plasmid has been constructed. After a plasmid harboring the cDNA gene fragment (pPICZaA-xynA) was obtained, the recombinant plasmid was linearized by SacI and introduced into *P. pastoris* X33 by electroporation, and transformants were selected on YPDS plates.

### DNA manipulation and transformation

DNA manipulation, plasmid isolation, and agarose gel electrophoresis were operated according to Sambrook et al. (2001). Transformation of *E. coli* was conducted according to Chung et al. (1989).

### Protein expression and purification

Unless otherwise noted, all purification steps were performed at 4°C. Single colonies were picked into 5 mL BMGY medium for preculture, and incubated them at 30°C overnight. After centrifugation at 6,000 rpm for 10 min, cells were transferred into 25 mL BMMY medium for induction and expression at 30°C for 3 d, and 250 µL methanol was added to medium 3 times a day. After expression, fermentation broth was centrifuged at 8,000 rpm for 10 min at 4°C. The supernatant containing the crude xylanase proteins was loaded onto Ni<sup>2+</sup>-NTA column. Bind Resins (Novagen, Madison, WI, USA) equilibrated with 1×Ni<sup>2+</sup>-NTA Bind Buffer. The wash and elution procedures were performed according to the instructions provided by the manufacturer. The purified protein was stored at -80°C before SDS-PAGE and analysis of its biochemical properties. Protein concentrations were determined by the Bradford assay (Bradford 1976), using bovine serum albumin as a standard.

### Xylanase activity assays

The xylanase activity was determined by measuring the release of reducing sugars from oat spelt xylan (1%, w/v) using the dinitrosalicylic acid method (Miller, 1959). Reaction mixture containing 1 ml of a solution of 1% oat spelt xylan in citrate buffer 50 mM, pH 5.0 plus 1 ml of the diluted purified enzyme, was incubated for 30 min at 50°C. One unit of xylanase was defined as the amount of enzyme required to release 1 µmol of xylose from xylan in 1 min under the assay condition.

### Sequence submission

The nucleotide sequences were deposited in the GenBank database under accession numbers EU846237 and EU846238.

## RESULTS

### Identification of strain *Aspergillus* sp. SH-2016 producing xylanase

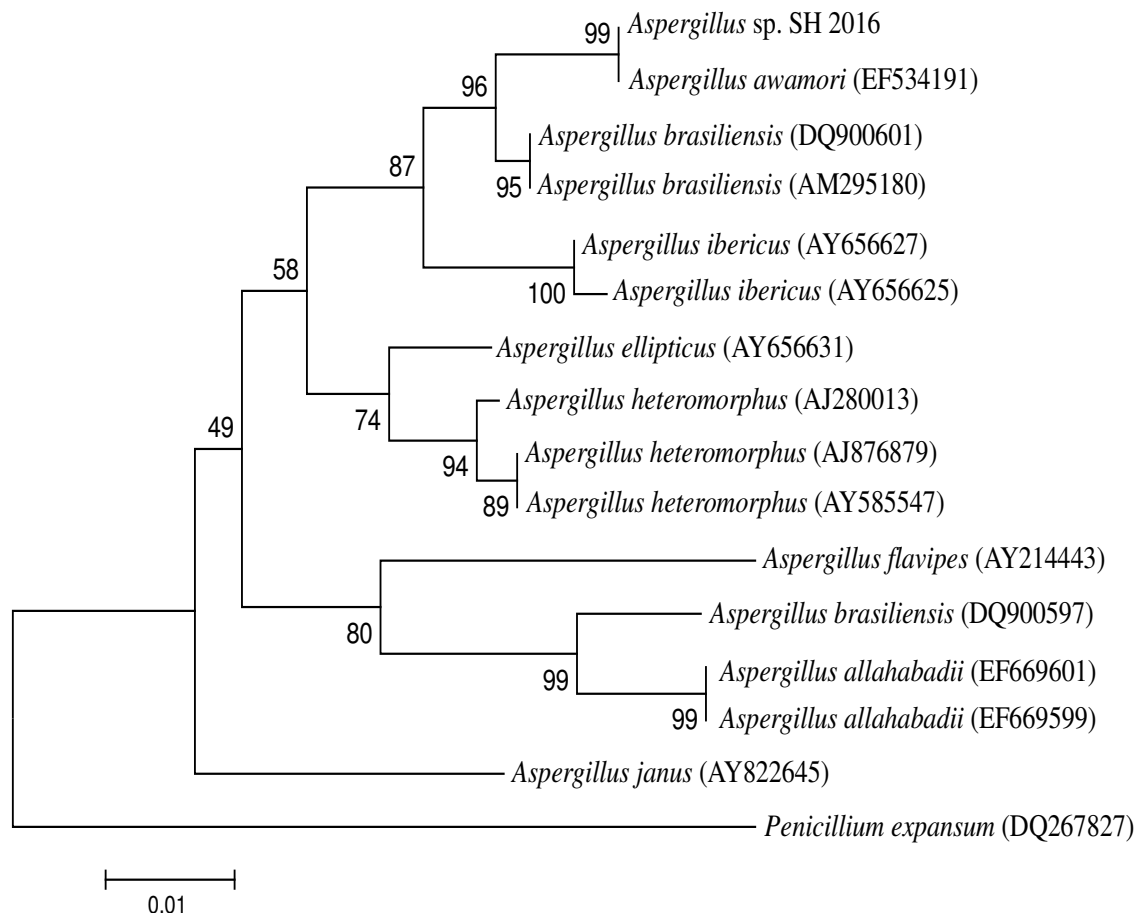
The identification of *Aspergillus* sp. SH-201 was according to the analysis of the detailed morphological and physiological properties, and the ITS rDNA gene sequence (GenBank accession no. EU846237). The analysis results showed that this strain was found to be similar to *A. awamori* (homology, 99%/582 bps, based on ITS rDNA). The approximate phylogenetic position of the strain is shown in Figure 1. According to the physiological and biochemical characterization as well as the comparison of ITS rDNA gene sequence, the strain *Aspergillus* sp. SH-2016 was identified as a strain of *A. awamori*, and named *Aspergillus awamori* SH-2016.

### RT-PCR and cDNA amplification

The products amplified with the primers 5'-GCTCCTGTGCCGGAACCTG-3' and Oligo(dT)15 were submitted to the agarose gel electrophoresis. The fragments were then recovered and ligated with pMD18-T, and the recombinant plasmid, named pMD18-T-xynA, was introduced into the competent cell *E. coli* JM109. A number of positive clones (colorless) were obtained. Among these, three were picked to prepare the single-strand for sequence analysis. As a result a sequence of about 1.6 kb was achieved. The sequence analysis using software DNAMEN (Version 4.0, Lynnon Biosoft, Canada) indicated that the fragment amplified contained a complete open reading frame (ORF) of 591 bp encoding 196 amino acids. Homology comparison revealed that its sequence (Figure 2) was similar to that of some of the most reported xylanase genes (Genebank accession No. X78115 and No. D14848). Alignment result also suggested that the part of cDNA not containing the upstream sequence has been obtained. However, the objective, cloning the structural cDNA gene encoding xylanase, has been reached. This method is easier and cheaper than the construction of cDNA library. Subsequently, a pair of primers with EcoRI and XhoI site was designed respectively and PCR amplification was conducted adopting the recombinant plasmid pMD18-T-xynA as template, and the resulting PCR products were submitted to agarose gel (Figure 3) and sequenced.

### Expression and purification of xylanase

Many proteins require formation of stable disulfide bonds to fold properly into a native conformation. Without the formation of stable disulfide bonds, these proteins may be degraded or accumulated as inclusion bodies. It is not easy to express the eukaryotic gene in prokaryotic host. Even if the gene can be expressed, the products are not



**Figure 1.** The phylogenetic dendrogram for *Aspergillus* sp. SH 2016 and related strains based on the ITS rDNA sequence. Numbers in parentheses are accession numbers of published sequences. Bootstrap values were based on 1000 replicates. *Penicillium expansum* was used as the outgroup.

always soluble and in an active form in endocellular environment. To gain the active xylanase in the heterogeneous host, we expressed the xylanase in *P. pastoris*. At the same time, pPICZaA, the vector with  $\alpha$  factor signal sequence, was used for the expression. The products expressed in host could be transported into medium, and the signal sequence was cut down by the signal identification system of host after the products were expressed, then the protein refolded to the active form. The structural gene encoding xylanase (GenBank accession number EU846238) was treated with the appropriate restriction endonuclease and subcloned into an expression vector pPICZaA. The recombinant plasmid map was shown in Figure 4. These plasmids were then introduced into competent cells of *E. coli* JM109, and many clones were picked up. After extraction, the recombinant plasmids containing the structural gene encoding xylanase was sequenced. The results of sequencing the recombinant plasmid indicated that the structural target gene has been obtained. Finally the plasmid pPICZaA-xynA was linearized by Sac I and introduced into *P. pastoris* X33, and several positive

transformants were obtained on the selective medium plates. The single colonies were transferred into BMMY medium, after incubation for about 24 h, and then the cells were collected by centrifugation at 6,000 rpm for 5 min. The cells were resuspended into the BMGY medium and induced by 250  $\mu$ l methanol three times a day. After induction, the supernatant were collected by centrifugation at 6,000 rpm and the activity of expressed xylanase was determined, and the results were shown in Table 1, which illustrated that the active xylanase was successfully expressed in this study. After expression, xylanase were purified using Ni<sup>2+</sup>-NTA column. The enzyme was stable in storage at 4°C in 20% glycerol for several weeks. The purified xylanase gave only one band on the SDS-PAGE, with a molecular weight of about 21 kDa (Figure 5).

#### Effect of pH on xylanase activities

The identical units of purified enzymes were dissolved in different buffers to achieve an initial activity for 30 min,

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1      ATGGCACCTGTTCTCTGAGCCTGTTTTAGTTTCAAGATCTGCTGGCATTAAATTACGTACAA
1      M A P V P E P V L V S R S A G I N Y V Q
61     AACTACAACGGTAATCTAGGTGATTTCACTTACGATGAAAGTGCCGGCACATTTTCAATG
21     N Y N G N L G D F T Y D E S A G T F S M
121    TATTGGGAGGATGGCGTTTCTTCCGATTTTGTGCGTTGGATTGGGTTGGACAACAGGTTCA
41     Y W E D G V S S D F V V G L G W T T G S
181    AGTAACGCTATCACCTATTTCAGCAGAGTACTCAGCCAGTGGCTCTTCATCTTACCTTGCC
61     S N A I T Y S A E Y S A S G S S S Y L A
241    GTATATGGGTGGGTGAACATCCACAAGCCGAGTATTACATAGTGGAGGATTACGGAGAT
81     V Y G W V N Y P Q A E Y Y I V E D Y G D
301    TACAACCCCTGCAGTTCGCAACTAGTCTTGGAAGTGTCTACTCAGACGGATCTACTTAC
101    Y N P C S S A T S L G T V Y S D G S T Y
361    CAAGTCTGTACAGATACTAGAACTAATGAGCCATCCATCACAGGTACTTCCACGTTTACC
121    Q V C T D T R T N E P S I T G T S T F T
421    CAATACTTCTCTGTTAGAGAGTCCACGAGAACGTCCGGCACGGTTACCGTAGCTAACCAC
141    Q Y F S V R E S T R T S G T V T V A N H
481    TTTAATTTCTGGGCCCAACATGGATTGGTAACAGTGATTTCATTACCAGGTGATGGCT
161    F N F W A Q H G F G N S D F N Y Q V M A
541    GTTGAAGCCTGGAGTGGCGCAGGTTTCAGCCTCCGTTACAATTTTCATCATAG
181    V E A W S G A G S A S V T I S S *

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**Figure 2.** Nucleotide sequence of xylanase and its deduced amino acid.

after which the relative residual activity was measured to assess the effect of pH with standard assay conditions. In this experiment, buffers including citric acid- $\text{Na}_2\text{HPO}_4$  (pH 3.4 - 8.0) and Tris-HCl (pH 7.2 - 9.1) with concentrations of 100 mM for each were used. After incubation at different pH buffers, the activity of xylanase could be detected at pH ranging from 3 - 8, while the maximum relative activity of xylanase was observed at pH 4.0. The detailed results were shown in Figure 6.

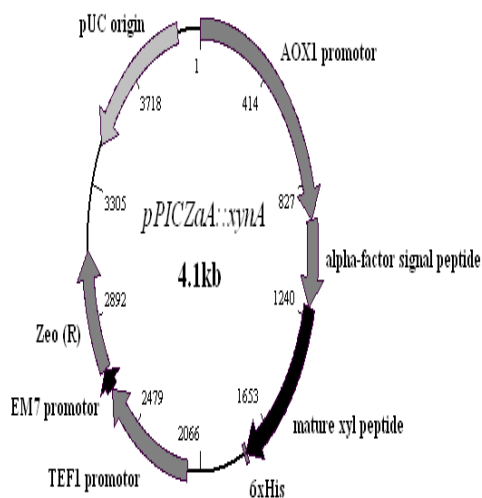
#### Effect of temperatures on xylanase activities

To evaluate the effect of temperature on the enzyme activity, the enzyme was pretreated in a preheated water

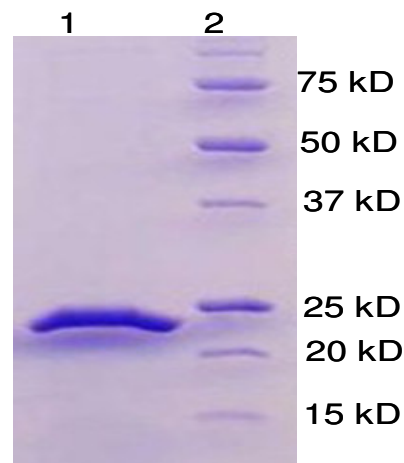
bath at various temperatures ranging from 20 to 85°C, the relative residual activities were assayed with standard assay conditions. The activities of enzymes that assayed under the standard reaction were taken as 100%. Xylanase activities slowly decreased from 20 to 60°C and dropped significantly at 70°C, it almost lost all of its activity at 85°C. Thus, the enzyme was sensitive to heat. The detailed was shown in Figure 7.

#### Effect of metal ions and other reagents on xylanase activity

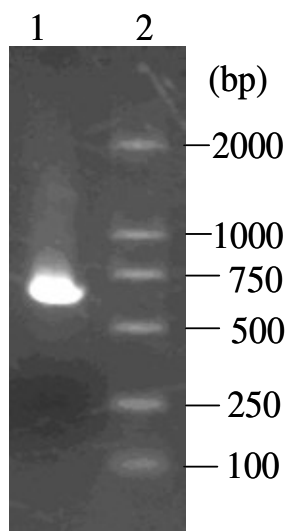
The effects of different metal ions and other reagents on the activities of purified xylanase were examined by incu-



**Figure 3.** Schematic diagram of expression construct.



**Figure 5.** SDS-PAGE analysis of purified xylanase. 1. The purified xylanase. 2. Molecular mass marker proteins.

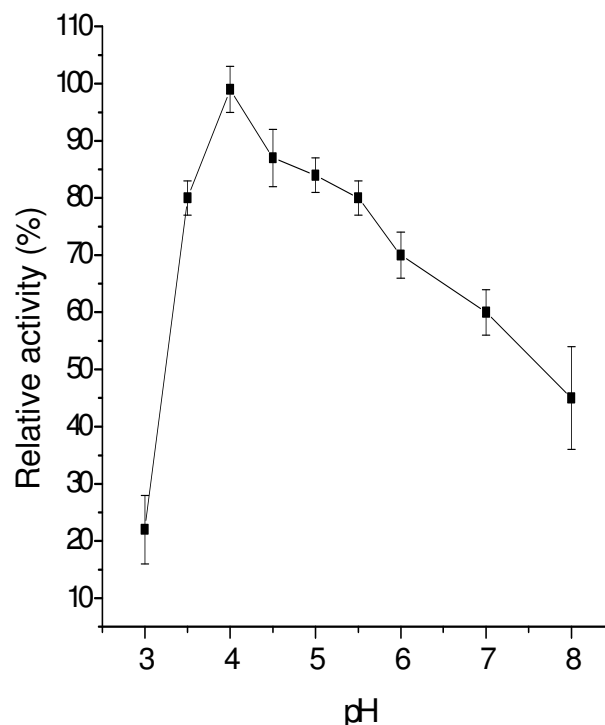


**Figure 4.** Agarose gel electrophoresis of resulting PCR fragment (Lane 1). Lane 2: Molecular weight

bating the enzymes in the presence of the reagents at 40°C for 1 h. The residual activity was assayed by the standard method. Activities of purified xylanase were inhibited by several metal ions, such as  $\text{Hg}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Ag}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Mn}^{2+}$  and  $\text{Zn}^{2+}$ . However, the  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  can activate the xylanase. The xylanase were greatly affected by EDTA with 83.4% inhibition. The detailed results were shown in Table 2.

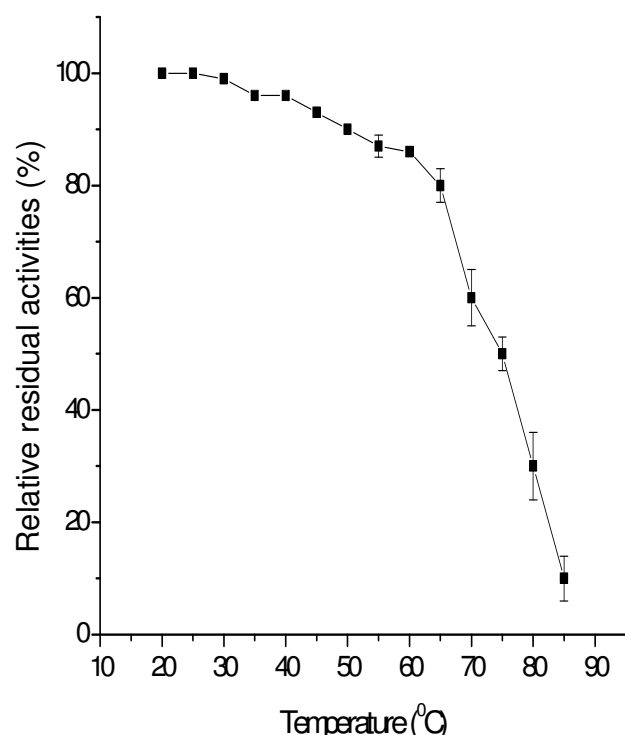
#### Determination of the kinetic parameters

The Michaelis constant  $K_m$  values and  $V_{max}$  for xylanase were determined by assaying purified enzyme



**Figure 6.** The influence of pH on xylanase activity.

at increasing spelt xylan concentrations ranging from 0.5 to 8 mg. The temperature, pH and quantity of the enzyme were kept the same as the standard enzyme activity assay described above. The values of  $K_m$  and  $V_{max}$  for xylanase were calculated according to Lineweaver-Burk plots (Fromm 1979). The values of  $K_m$  and  $V_{max}$  were calculated to be 6.6 mg  $\text{ml}^{-1}$  and 982  $\mu\text{mol}$  reducing sugar as xylose  $\text{min}^{-1}$   $\text{mg}^{-1}$  protein, respectively, for hydrolysis of spelt xylan.



**Figure 7.** The influence of temperature on xylanase activity.

**Table 1.** Activity of recombinant xylanase.

Strains/plasmid/inducer	Activity(U/mL)
<i>P. pastoris</i>	0
<i>P. pastoris</i> /pPICZaA-xynA	10.08
<i>P. pastoris</i> /pPICZaA-xynA/methanol	121.24
<i>P. pastoris</i> /pPICZaA-xynA/methanol	109.31

## DISCUSSION

Xylan is the second most abundant biopolymer after cellulose and the major hemicellulosic polysaccharide found in the plant cell wall (Timell, 1967). Xylanases have attracted considerable research interest because of their potential industrial applications, for examples, xylanases has been used for improving the quality and texture of bakery products, reducing the amount of chlorine required for bleaching of paper pulp, and increasing the nutritive quality of poultry diet (Gilbert and Hazlewood, 1993).

This study was aimed at isolating strains capable of producing xylanase for industrial purpose. Various microbial strains were isolated from soil samples by the enrichment culture. The strain, *A. awamori* SH-2016, which produce the maximum xylanase, was isolated and selected as a best strain for further studies. Generally, microorganisms are identified mainly using morphological characteristics. However, these methods of identification

**Table 2.** Effect of metal ions and some chemicals on xylanase activity\*.

Chemicals	Concentration (mM)	Relative activity (%)
None		100
EDTA	1	16.6
CoCl <sub>2</sub>	1	98.7
FeCl <sub>2</sub>	1	71.33
CaCl <sub>2</sub>	1	115.33
MgCl <sub>2</sub>	1	110.76
MnSO <sub>4</sub>	1	74.54
ZnSO <sub>4</sub>	1	98.42
AgNO <sub>3</sub>	1	33.9
Hg <sub>2</sub> Cl <sub>2</sub>	1	26.5
CuSO <sub>4</sub>	1	90.25

\*Enzymes activities were determined in the presence of an additional test chemical substance under the standard assay conditions.

are often problematic, as there can be different morpho/biotypes within a single species. They are also time-consuming, and also require a great deal of skill. DNA sequence analysis methods are an objective, reproducible, and rapid means of identification, therefore, they have been widely used. For the molecular approach, our strategy was to identify these mycelia sterilia by means of rDNA sequence comparison, as well as phylogenetic analysis conducted in several stages of varying taxonomic resolutions (Liu et al., 2008).

The analysis of the ribosomal region via PCR has been widely employed for characterizing different fungal species (Gardes and Bruns, 1993). In both vegetal and fungal organisms the structure of the ribosomal region comprises repeated clusters of coding regions (Erland et al., 1994). The ITS regions separating genes 17 S and 25 S, can be amplified by specific primers anchored in these two units. Since the ITS region is highly conserved, it is variable between different species. ITS region polymorphism is often used in taxonomy, but now, it is being well recognized for identifying fungi species. In this study, we combined the morphological and physiological methods with DNA sequence analysis to identify the genetic position of the isolated strain producing xylanase. To date, many xylanases have been isolated and characterized (Ghosh et al., 1993; Haltrich et al., 1996; Ito, 1993; Ziaie-Shirkolaei et al., 2008). In addition, some of the molecular biology of these enzymes has been studied (Alam et al., 1994; Eliger et al., 1994; Patel and Ray, 1994; Tsujibo et al., 1990; Wang et al., 1993; Moreau et al., 1994; Kulkarni et al., 1999). In this study, a newly isolated and identified strain, *Aspergillus awamori* SH-2016, is capable of producing xylanase, was obtained, and the gene encoding xylanase was successfully cloned and expressed, which would enrich the resource of xylanase.

Several ions could influence the xylanase activity, which indicate metal ions have a stabilizing effect on the xylanase when it catalyzes the reactions. One of the interesting properties of this enzyme is that the optimal pH for the recombinant xylanase was about pH 5.0 which make it efficiently hydrolyze oat spelts at pH 4.5. This pH for the optimal enzyme activity of the purified xylanase is in the range of that reported for other fungi and bacteria (Wong et al., 1988). This again suggests that the xylanase from *A. awamori* SH-2016 could be of commercial interest for the digestion of residual xylan in the animals' stomachs under acidic conditions. At the same time, the enzyme was sensitive to heat. To improve the thermostability and activity of xylanase for industrial applications, the xylanase could be modified by rational design of proteins or by directed molecular evolution of proteins (Miyazaki et al., 2006; Liu et al., 2006), which are ongoing in our further studies. Characterization experiments yielded a great deal of information regarding the biochemical nature of this xylanase, which offered a possibility for this xylanase with potential industrial application.

Many industrial types of yeast have been developed as recombinant systems for the commercial production of xylanase (Damaso et al., 2003). These organisms combine ease of genetic manipulation with the ability to perform many eukaryotic post-translational modifications (Cereghino et al., 2002). One of the most commonly used systems is the yeast *Pichia pastoris*. This organism possesses a number of attributes that make it an attractive host for the expression and production of xylanase (Berrin et al., 2000). Most importantly, it can be grown conveniently in a simple and inexpensive medium and it does not secrete any endogenous xylanase of its own (Berrin et al., 2000). The xylanase from *A. awamori* SH-2016 was successfully cloned and expressed in *Pichia pastoris* in this work. Overexpression of this kind of xylanase would facilitate better understanding of the structure of the enzyme and could result in the production of large amounts of efficient xylanase biocatalyst.

## ACKNOWLEDGEMENT

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