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*Full Length Research Paper*

# Purification and characterization of an extracellular xylanase from *Aspergillus niger* C3486

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**An extracellular xylanase from *Aspergillus niger* C3486 grown on a medium containing D-xylose was purified to homogeneity as indicated by disc acrylamide gel electrophoresis with an apparent molecular mass of about 25 kDa using DEAE-Sephadex A-50 and Sephadex G-100 column chromatography. The xylanase was purified 14.79 fold with 29.88% recovery. Optimal temperature and pH for activity was observed at 55°C and 5.5, respectively. The extracellular purified xylanase had $K_m$ value and $V_{max}$ of 0.104 mg/ml and 24.8 µmol $min^{-1}$ $mg^{-1}$, respectively. The metal ions $Hg^{2+}$ and $Cu^{2+}$ showed some inhibition effects, while $Mg^{2+}$ and $Cr^{3+}$ had small stimulating effects on the activity. The xylanase only showed activity on the xylan, and the zymogram analysis indicated that this enzyme was an active xylanase. N-terminal amino acid sequence analysis indicated that the first 18 amino acid residues of N-terminal sequence of the enzyme were PVLVSRSAGINYVQNYNG. Enzyme modification showed that tryptophan was in the active site of the enzyme.**

**Key words:** *Aspergillus niger* C3486, xylanase, purification, characterization.

## INTRODUCTION

Xylan, the major hemicellulose component in a plant cell wall, is easily found in solid agricultural and agroindustrial residues, as well as in effluents released during wood processing (Collins et al., 2005; Anthony et al., 2005). Frequent inappropriate discarding of xylan caused great damage to the ecosystem (Prade, 1995). Xylanases are the key enzymes, which play important roles in the hydrolyzation of xylan (Vardakou et al., 2008; Collins et al., 2002), and therefore it is important in biopulping wood, bleaching pulp, treating animal feed, and bioconversion of lignocellulose materials to fermentative products. Further, the xylanases are especially used in enzymatic treatment of kraft pulp before bleaching in the paper industry, which has a positive impact on the environment.

In recent years, many kinds of xylanases have been isolated from various microorganisms (Stricker et al., 2008; Canakci et al., 2007). Filamentous fungi such as *Aspergillus* spp. and *Trichoderma* spp. are of particular interest, and many microbial xylanases from fungi and bacteria have been purified and characterized (Manimaran and Vatsala, 2007). Various thermostable xylanases have been isolated from cultures of *Schizophyllum commune* (Katarina et al., 2005) and *Bacillus* spp. (Okazaki et al., 1985).

Although xylanases have been investigated for many years, many industrial xylanases show a low activity (Lee et al., 2007). In our previous studies, xylanase-producing strain *Aspergillus niger* C34 was mutated by γ-radiation and diethyl sulfate (DES), and a high xylanase-producing strain named *A. niger* C3486 was obtained (Wang and Hu, 2005). The aim of this study was to purify and characterize the extracellular xylanase produced by *A. niger* C3486.

## MATERIALS AND METHODS

### Chemicals

Larch wood xylan, N-Bromosuccinimide (NBS), p-Hydroxymercuri-

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benzoate (PHMB), 3,5-dinitrosalicylic acid (DNS), BSA and Protein marker were obtained from Sigma Chemical Co., USA. DEAE sepharose was purchased from Pharmacia Fine Chemicals, Uppasala, Sweden. All the other chemicals used were of analytical grade unless otherwise stated.

**Microorganism**

*A. niger* C3486 was obtained from the culture collection of our laboratory, details in the methods relating to culture collection were carried out as previously described (Wang and Hu, 2005).

**Medium and cultivation conditions**

For xylanase production, the strain *A. niger* C3486 was grown in the medium containing (g/L): Peptone 2, yeast extract 0.2, $MgSO_4$ 0.01, $(NH_4)_2SO_4$ 2.0, $K_2HPO_4$ 0.46, $KH_2PO_4$ 0.1 and D-xylose 5.0. Fermentation of the organism was carried out in a 5 L New Brunswick Microferm fermentor at 50°C in a rotatory shaker at 600 rpm with aeration of 8 to 10 L/min for 12 to 18 h.

**Enzyme purification procedure**

Ammonium sulfate was first added to 576 ml of culture supernatant containing xylanase to 40% saturation. After stirring overnight at 4°C, the precipitate was discarded by centrifugation (10000 g, 15 min). To the resultant supernatant, ammonium sulfate was added to give 75% saturation, and the mixture was stirred overnight at 4°C. The precipitate was collected by centrifugation, then resuspended in 0.067 mol/L sodium phosphate buffer (pH 5.5) and desalted by dialysis. The concentrated enzyme sample was loaded onto a DEAE-Sephadex A-50 column (2.5 cm × 20 cm), which had been equilibrated with 0.067 mol/L sodium phosphate buffer (pH 5.5). The protein was eluted with a linear gradient of 0 to 0.8 mol/L NaCl. Fractions (2.0 ml) were collected at a flow rate of 1 ml/min, and those with high xylanase activity were pooled. After desalting, the enzyme was loaded onto a Sephadex G-100 gel filtration column (1.5 cm × 45 cm) and fractions (2.0 ml) were collected at a flow rate of 1 ml/min. SDS-PAGE was carried out to estimate molecular mass.

**Xylanase activity assay**

Appropriately, diluted enzyme (0.2 ml) was mixed with 1 ml of 1% (w/v) birch wood xylan (molecular weight, 30,000.) and 1.8 ml of 0.067 M phosphate buffer (pH 5.5). The mixture was then incubated at 55°C for 10 min. The reducing sugar content of the mixture was determined by the DNS method (Miller, 1959). Control without enzyme was maintained throughout the investigation. One unit of xylanase activity was defined as the amount of enzyme which produces 1 µmol of xylose equivalent per minute.

**Effect of pH and temperature on xylanase activity and stability**

The enzymetic reactions on the effect of pH were carried out under standard assay conditions with 1 ml of 1% birch wood xylan dissolved in different buffers: Citric acid-$Na_2HPO_4$ (pH 4 to 8) for 10 min at 55°C. For pH stability, the purified enzyme (0.2 ml) was placed in the aforementioned buffers with different pH levels and incubated at 55°C for 90 min and the residual enzyme activity was then assayed as described. All of these assays were repeated three times and results are expressed as relative percentages compared with the highest value.

The effect of temperature on the reaction rate was determined by incubating the purified enzyme with the substrate at temperatures ranging from 30 to 85°C for 10 min under the standard assay conditions, and then the residual enzyme activity was checked. The thermostability assay was conducted by incubating the enzymes without the substrate at different temperatures (55, 60, 65 and 70°C) for different time intervals. Residual activity of the enzyme was assayed and expressed as percent activity.

**Effect of metal ions and EDTA on xylanase activity**

The effect of cations on enzyme activity was achieved by incorporating different mineral salts ($MgSO_4$, $CrCl_3$, $ZnCl_2$, $FeSO_4$, $AgNO_3$, $CoCl_2$, $MnSO_4$, $HgCl_2$, and $CuSO_4$) at a final concentration of 1 mM. The extracellular enzyme was preincubated with each of these metals. EDTA was also tested at a concentration of 3 mM at 55°C in 0.067 M phosphate buffer at pH 5.5 for 10 min. The residual activity was then checked under the standard assay conditions.

**Effect of organic solvents on xylanase activity**

Different organic solvents (ethanol, methanol, acetone, etc.) at different concentration gradients (0 to 30%) were added into the enzyme reaction system for 10 min, and the residual activity of the enzyme was quantified under the standard assay condition.

**Substrate specificity of xylanase and determination of kinetic constant**

Activity of the xylanase was tested as described by using 1% (w/v) low viscosity carboxymethyl cellulose (CMC), avicel, laminarin and xylan, respectively as substrates to determine the substrate specificity of the xylanase (Gashaw et al., 2006). $K_m$ and $V_{max}$ values of the xylanase were determined by measuring enzyme activity at various concentrations of xylan, and were calculated using Lineweaver-Burk equation method.

**Zymogram analysis**

The enzyme sample was subjected to electrophoresis on an SDS-PAGE containing 0.1% xylan. After electrophoresis, the gel was soaked in 25% (v/v) isopropanol with gentle shaking to remove SDS and renature the proteins in the gel. The gel was then washed four times for 30 min at 4°C in 0.1 M acetate buffer (pH 5.5). After further incubation for 1 h at 50°C, the gel was soaked in 0.1% Congo red solution for 30 min at room temperature and washed with 1 M NaCl until excess dye was removed from the active band. After being submerged in 0.5% acetic acid, the background of the gel turned dark blue and the activity bands were observed as clear colorless areas (Min-Jen et al., 2002).

**N- terminal amino acid sequence and enzyme modifications**

After SDS-PAGE, purified enzyme on polyacrylamide gel was transferred to a polyvinylidene difluoride membrane by electroblotting and stained with Ponceau S solution containing 5% acetic acid. The stained portion was excised and used for N-terminal sequencing directly. The N-terminal amino acid sequence of xylanase C3486 was determined with an Applied Biosystems model 475 A gas-phase sequencer (Fuzhou University, Fuzhou, China) (Hiroshi et al., 1992).

The modifier, NBS of 4 µM or PHMB of 1 mM was added to tubes containing 1 ml of 50 mM succinic acid buffer (pH 5.5) with different

**Table 1.** Purification result of xylanase from *Aspergillus niger* 3486. Values given are the averages of at least three experiments.

| Purification step | Total protein (mg) | Total activity (U) | Specific activity (U/mg) | Recovery (%) | Purification fold |
|---|---|---|---|---|---|
| Culture filtrate | 2118.58 | 17711.37 | 8.36 | 100 | 1.00 |
| $(NH_4)_2SO_4$ precipitation | 502.97 | 12951.38 | 25.75 | 73.12 | 3.08 |
| DEAE-Sephadex A-50 | 78.49 | 7128.83 | 90.82 | 40.25 | 10.86 |
| Sephadex G-100 | 42.79 | 5292.16 | 123.68 | 29.88 | 14.79 |

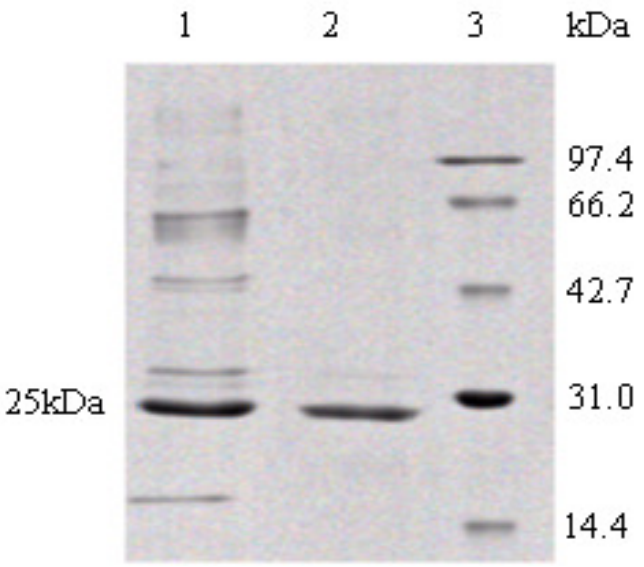


**Figure 1.** SDS-PAGE photograph of purified xylanase. 20 microliters of samples were loaded on to electrophoresis on an SDS-PAGE containing 0.1% xylan. Proteins were stained with colloidal silver. Lane 1**:** The fermented supernatant containing xylanase; Lane 2**:** Xylanase; Lane 3: Mid-range protein molecular.

amounts of xylan (0 to 5 mg), and the enzyme (1 µg) was then added to the tubes. The mixtures were incubated for 10 min at 55℃. The residual activity was then determined by adding 0.5% xylan to the reaction tubes under the standard assay condition.

## RESULTS

### Enzyme purification

Purification using DEAE-Sephadex A-50 column and Sephadex G-100 column chromatography resulted in one peak of xylanase activity, respectively. About 14.79-fold purification was achieved with 29.88% recovery of xylanase activity, yielding a specific activity of 123.68 U/mg protein. The purification result is shown in Table 1. SDS-PAGE of the purified enzyme revealed a single protein band, suggesting that this xylanase was formed by a single polypeptide chain with a molecular mass of about 25 kDa (Figure 1).

### Effect of pH and temperature on xylanase activity and stability

The enzyme was most active at a pH of about 5.5. The pH stability of the xylanase was performed by incubating the enzyme solution at different pHs at 55℃ for 90 min, and the residual activity was measured by the standard assay method. The result showed that the enzyme was stable at pH 4.0 and 5.0, and more than 80% of the activity after incubation for 90 min could be retained, while at pH 6.0, only about 50% activity could be retained in the same condition. However, at pH 8.0, a sharp decrease in activity was observed after 10 min (Figure 2A).

The optimum temperature for enzyme activity was tested at the temperature ranged from 30 to 85℃ in phosphate buffer (pH 5.5). The purified xylanase gave the highest activity at 55℃. The thermostability of the xylanase was measured by incubating it at different temperatures and the residual activity of various times was determined. Figure 2B showed that after 30 and 100 min of incubation at 55℃, the enzyme retained 100 and 70.89% of its activity, respectively. At 60℃, 85.18 and 60.11% of the activity were retained after 30 and 100 min of incubation, respectively. When preincubated at 70℃, however, the enzyme was not stable and rapid inactivation of the activity was observed.

### Effect of different metal ions and EDTA on activity

The effect of different reagents on the activity of xylanase is shown in Table 2. The metal ions $Cu^{2+}$ and $Hg^{2+}$ showed some inhibition of the activity, while $Mg^{2+}$ and $Cr^{3+}$ had small stimulating effects on the activity. The addition of EDTA affected the activity slightly. All these results meant that this xylanase was not a metalloenzyme.

### Effect of different organic solvents on activity

The influences of different organic solvents on xylanase activity are shown in Figure 3. Primary alcohols including methanol, ethanol and isopropanol as well as polyhydric alcohol containing glycol and glycerol, all showed inhibition effects on xylanase activity. When the

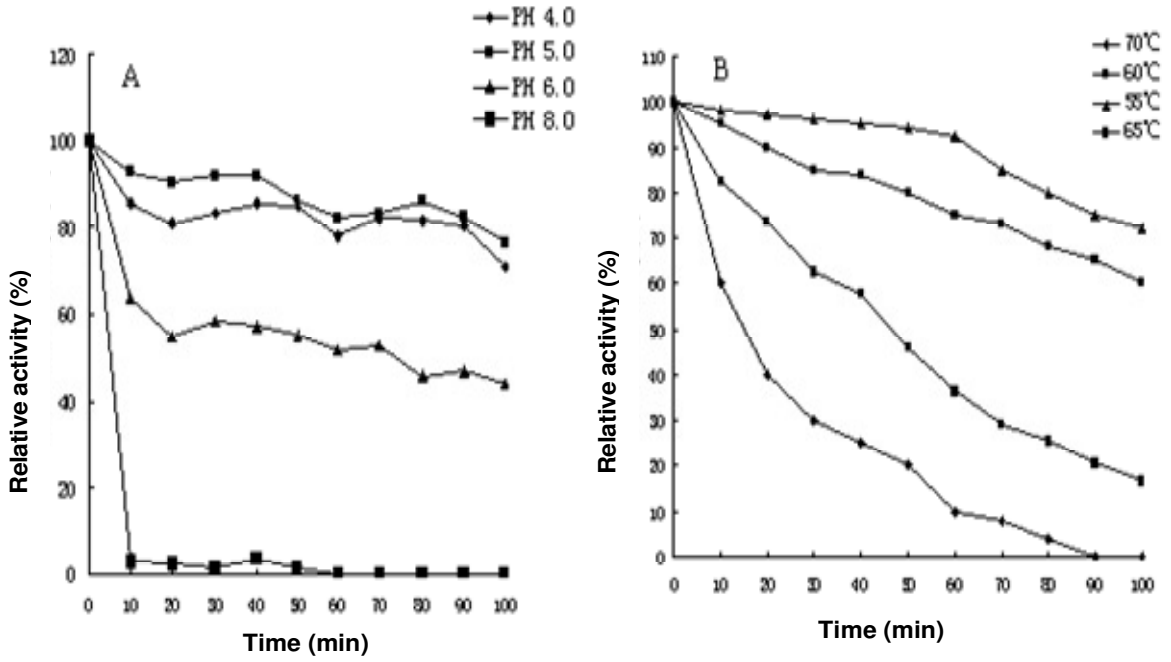


**Figure 2.** Effect of pH and temperature on xylanase stability. (A) Effect of pH on xylanase stability. The purified enzyme was preincubated in different buffers: citric acid-$Na_2HPO4$ (pH 4 to 8) for 90 min at 55℃ and the residual enzyme activity was assayed. (B) Effect of temperature on xylanase stability. The enzyme was preincubated at 55, 60, 65 and 70℃ in 0.067 M phosphate buffer (pH 5.5). At the indicated times, aliquots were withdrawn and the residual enzymatic activity was measured under the standard assay conditions, as expressed as percentage activity of zero time control of untreated enzyme. Values given are the averages of at least three experiments.

**Table 2.** Effects of different metal salts and EDTA on enzyme activity values given are the averages of at least three experiments.

| Compound | Concentration (mM) | Relative activity (%) |
|---|---|---|
| $MgSO_4$ | 1 | 106.2 |
| $CrCl_3$ | 1 | 108.9 |
| $ZnCl_2$ | 1 | 98.4 |
| $FeSO_4$ | 1 | 97.5 |
| $AgNO_3$ | 1 | 96.3 |
| $CoCl_2$ | 1 | 92.6 |
| $MnSO_4$ | 1 | 78.4 |
| $HgCl_2$ | 1 | 20.3 |
| $CuSO_4$ | 1 | 33.6 |
| EDTA | 3 | 86.3 |

concentrations of these alcohols were less than 10% (v/v), the xylanase activity decreased significantly as the concentration increased. But when the concentrations were above 10% (v/v), the relative activity was decreased slowly with an increase in the concentration. Among these results, the effect of the isopropanol was more complicated. When the concentration reached 10%, the relative activity of xylanase was less than 15%. However, when the concentration increased to 15%, the relative activity rebounded to 66%. The effects of the methanol, glutaraldehyde and acetone on the xylanase activity are shown in Figure 4, showing that these organic solvents all inhibited xylanase activity. Figure 5 indicates that both dimethyl sulfoxide (DMSO) and N,N-dimethylformamide (DMF) performed inhibition effect on the xylanase activity. However, the xylanase was more sensitive to DMF than to DMSO.

## Substrate specificity and kinetic constants

The substrate specificity of the enzyme was determined by performing the assay with different substrates. There was no detectable activity on CMC, avicel and laminarin. The enzyme only showed activity on the xylan.

Xylanase hydrolyzes birch wood xylan to release reducing sugars. At 55℃, the release of reducing sugar was linear with time and proportional to enzyme concentration. Kinetic experiments at 55℃ with different xylan concentrations gave $K_m$ and $V_{max}$ values of 0.104 mg/ml and 24.8 μmol $min^{-1}$ $mg^{-1}$, respectively.

## Zymogram analysis

The purified protein showed a clear band on the zymogram gel, detected by Congo red staining

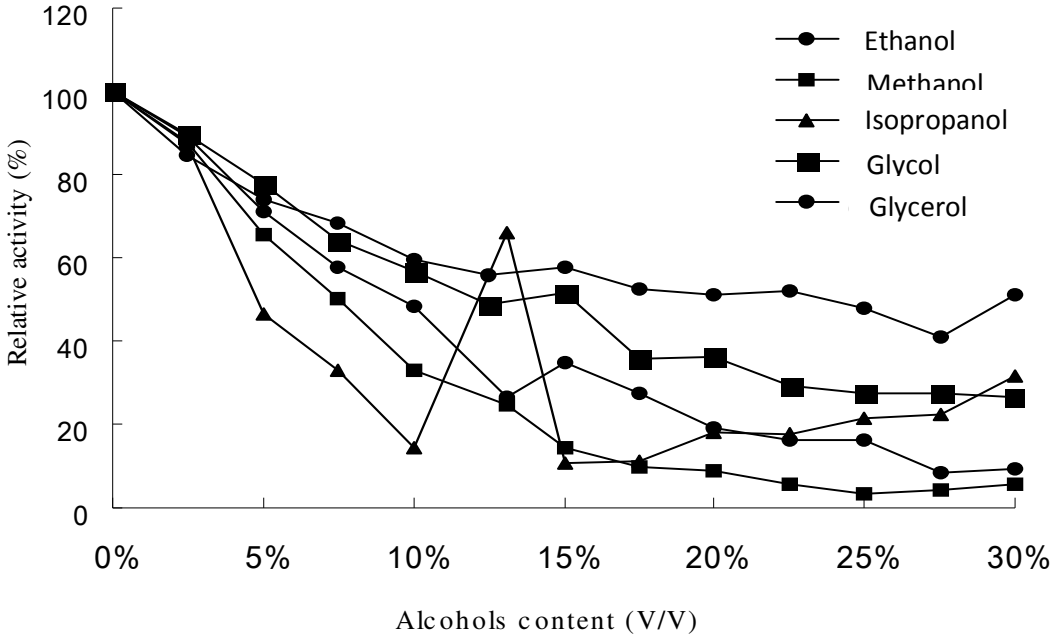


**Figure 3.** Effect of different alcohols on xylanase activity. The enzyme was incubated at 55℃ for 10 min in 0.067 M phosphate buffer at pH 5.5. The enzyme activity was measured in the presence of ethanol, methanol, isopropanol, glycol and glycerol. Values given are the averages of at least three experiments.

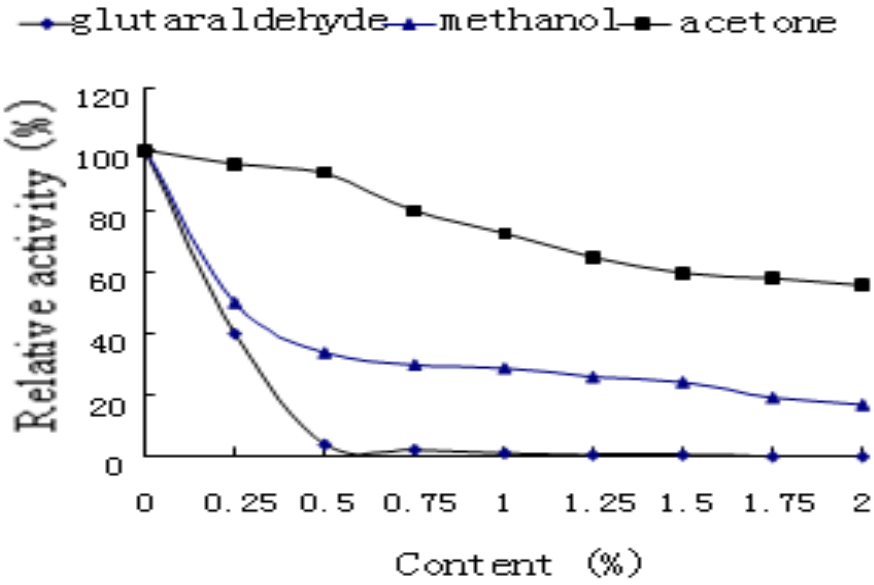


**Figure 4.** Effect of methanol, glutaraldehyde and acetone on xylanase activity. The enzyme was incubated at 55℃ for 10 min in 0.067 M phosphate buffer at pH 5.5. The enzyme activity was measured in the presence of methanol, glutaraldehyde and acetone. Values given are the averages of at least three experiments.

corresponding to the 25 kDa observed in lane 1 (Figure 6), indicating that the protein was an active xylanase. But the brightness of the visible active band did not quantitatively correspond to its xylanase activity (data not shown). On the other hand, the majority of low-molecular-mass xylanases remained in the supernatant after $(NH_4)_2SO_4$ fractionation. Thus, the ammonium sulfate fractionation seemed to be an effective method for

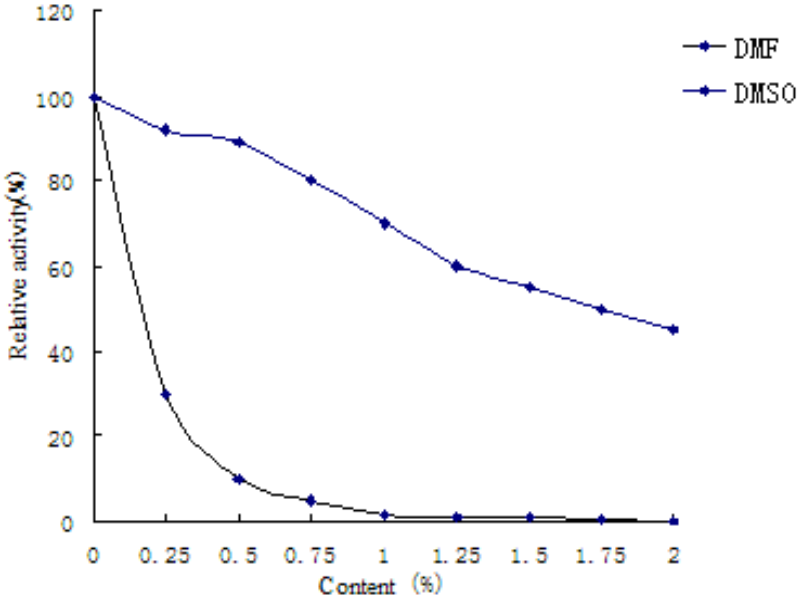


**Figure 5.** Effect of DMF and DMSO on xylanase activity. The enzyme was incubated at 55$^0$C for 10 min in 0.067 M phosphate buffer at pH 5.5. The enzyme activity was measured in the presence of DMF and DMSO (0 to 2%). Values given are the averages of at least three experiments.

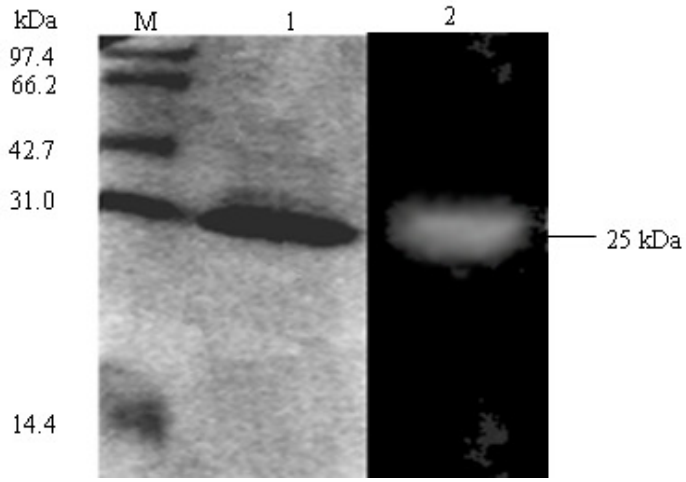


**Figure 6.** Zymogram analysis of xylanases from *A. niger* C3486. Lane M: Mid-Range protein Molecular; Lane 1: The purified xylanase; Lane 2: Zymography using Congo red-stained xylan. The corresponding clear band showed the xylanase activity.

removing low-molecular-mass xylanases.

### N-terminal amino acid sequence and chemical modifications of xylanase

N-terminal amino acid sequencing of the purified enzyme identified the first 18 residues: PVLVSRSAGINYVQNYNG. A search for homogies with other proteins was made through the National Center for Biotechnology Information (NCBI) using BLAST network service. This xylanase could probably be assigned to family G/11 based on its high homology to several xylanases of this family.

To test if the active site of the xylanase contained tryptophan (or cysteine), which was often shown to be involved in the active site of different xylanases (Alexander et al.,1993), we used NBS and PHMB as trytophan modifier and cysteine modifier, respectively. We also examined the ability of xylan to protect the enzyme from modifiers using different concentrations. The results showed that 1 mg xylan was needed to give 100% protection against inactivation by 4 µM NBS, which could completely inhibit xylanase (1 µg/mL). However, only about 8% inhibition was detected after treatment with 1 mM of PHMB. These results indicating that the presence of tryptophan is essential for the activity.

## DISCUSSION

Xylanase possessing high activity towards birch wood xylan has been purified and characterized from the culture supernatant of *Aspergillus niger* C3486. Unlike other microorganisms that produce multiple xylanases (Saha et al., 1999), only one xylanase was isolated from this strain.

The small $K_m$ value (0.104 mg/ml) and the highly specific activity of this enzyme might be an attractive property for biotechnological application of this enzyme. The low apparent mass (25 kDa) of this enzyme was

similar to that of xylanases from *Streptomyces lividans* 66 (31 kDa) (Kluepfel et al., 1990) and *Streptomyces roseiscleroticus* (22.6 kDa) (Grabski et al., 1991). This group of xylanase has been assigned to the category of low $M_r$ basic xylanases, in contrast to high $M_r$ acidic types of xylanases (Li et al., 1993). The presence of only one band on zymogram of xylanase on native gel indicated the presence of a single active xylanase.

Most xylanases known today are active at acidic pHs (Alexander et al., 1993), the xylanase in the present study also had an acidic optimum pH of 5.5 and was stable under acidic conditions.

Thermal stability testing showed that the activity reduced markedly when the temperature increased to more than 60°C. These properties should make it a good candidate in various industrial applications. For instance, with its pH stability and moderate thermal stability properties, it was most suitable for use in the animal feed industry (Chantasingh et al., 2006).

By testing the xylanase on various substrates, it was found that the enzyme was active on xylan and inactive on CMC, avicel and laminarin tested. These results showed that this xylanase was specific for hydrolyzing natural xylan and was free of cellulase activity, which was a desirable property for biobleaching of pulps. Xylanase activity was assayed in the presence and absence of metal ions and a metal chelator (EDTA). $Hg^{2+}$ and $Cu^{2+}$ inhibited the activity of the xylanase enzyme, which were similar to those concerning the xylanase reported by others (Collins T et al., 2002; Sandrim et al., 2005). However, $Mg^{2+}$ and $Cr^{3+}$ had small stimulating effects on the activity, in contrast, it has been reported that xylanase from the *Paenibacillus* sp. KIJ1 was slightly inhibited by $Mg^{2+}$ (Park and Cho, 2010). Nevertheless, cysteine was not detected in enzyme active center by chemical modification analysis, and the slight inhibition might be due to the reaction of this modifier with other residues.

It was reported that some enzymes performed high thermal stability and high catalytic activity in organic solvents (Klibanov, 1989). Later, a breakthrough has been made in the enzyme catalysis field, giving the birth of a new subject named nonaqueous enzymology. In order to find some activators of the xylanase, in this study, we selected several different organic solvents to test their functions on the xylanase activity. But the results showed that the organic reagents used in our study inhibited the xylanase activity. All these findings will give new insights to understand xylanases and their enzymatic applications. Future scientific works such as gene cloning, co-evolution by the technology of Error PCR and DNA Shuffling will be planned to maximize catalytic efficiency and productive yield of the enzyme.

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