Mycobiology 33(2): 84-89 (2005)

Production of Xylanolytic Enzyme Complex from *Aspergillus flavus* using Agricultural Wastes

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(*Received Januray 11, 2005*)

Five types of agricultural wastes were used for the production of xylanolytic enzyme by *Aspergillus flavus* K-03. All wastes materials supported high levels of xylanase and β-xylosidase production. A high level of proteolytic activity was observed in barley and rice bran cultures, while only a weak proteolytic activity was detected in corn cob, barley and rice straw cultures. Maximum production of xylanase was achieved in basal liquid medium containing rice barn as carbon source for 5 days of culture at pH 6.5 and 25°C. The xylanolytic enzyme of *A. flavus* K-03 showed low thermostability. The times required for 50% reduction of the initial enzyme activity were 90 min at 40°C, 13 min at 50°C, and 3 min at 60°C. Xylanolytic activity showed the highest level at pH 5.5~10.5 and more than 70% of the original activity was retained at pH 6.5 and 7.0. The higher stability of xylanolytic enzymes in the broad range of alkaline pH is useful for utilization of the enzymes in industrial process requiring in alkaline conditions. Moreover, the highest production of xylanolytic enzyme was obtained when 0.5% of rice bran was supplied in basal liquid medium. SDS-PAGE analysis revealed a single xylanase band of approximately 28.5 kDa from the culture filtrates.

KEYWORDS: Agricultural wastes, *Aspergillus flavus*, Rice bran, Xylanolytic enzyme

General agricultural wastes comprise cellulose, hemicellulose and lignin, among which hemicellulose is the second most abundant fraction available in nature (Taiz and Honigman, 1976). Xylan that is major component of the hemicellulose occupies the large portion of plant cell walls in nature and it consists of xylose. Recently, the attention of xylanolytic enzyme has dramatically increased due to their use in the industries of paper, food and textile with reduced the prime cost (Haltrich *et al*., 1996; Kulkarmi *et al*., 1999). For this reason, xylan degradation has been carried out by xylanolytic enzymes produced by a variety of microorganisms including bacteria, yeasts, and fungi (Carmona *et al*., 1997; Coughlan and Hazlewood, 1993; Kadowaki *et al*., 1995). Especially, filamentous fungi receive much attention for their potential applications as xylanolytic enzyme producers because fungi produced more xylanolytic enzymes than yeasts or bacteria (Coelho and Carmona, 2003). Moreover, Haltrica *et al*. (1996) and Bhat (2000) tried to obtain xylanolytic enzyme from the genera *Trichoderma* and *Aspergillus* for commercial use. Nevertheless, only a few cases have been reported for the production of xylanolytic complex by *A. flavus* using agricultural wastes, except for a strain reported by Abdel-Stater and El-Said (2001).

In this study, I determined the effect of different agricultural wastes as carbon sources on the production of xylanolytic complex by *A. flavus*. Also, I investigated the influence of physical and chemical factors related to cultural conditions for xylanolytic enzyme production by this fungus, and partially characterized the xylanolytic enzyme. The results in this study would enable to develop a simple culture medium using cheap ingredients for reduction the prime cost in enzyme production.

Materials and Methods

Strains and media. *Aspergillus flavus* K-03 used in this work was isolated from soil in a screening program for protease producing microorganisms (Kim, 2003). It was maintained on slants of potato dextrose agar (PDA) at 30°C for 5 days for spore production. The conidial suspensions were prepared by adding 10 *ml* of sterilized water to slant cultures. Liquid cultures were carried out in the basal liquid medium (0.5 g $NaNO_3$, 1.0 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$ and 0.01 g $FeSO_4 \cdot 7H_2O$ per liter) containing 5 g of lignocellulosic substrates such as rice bran, barley bran, corn cob, rice straw, and barley straw. The pH was adjusted to 7.0 with 1 M NaOH prior to autoclaving (121°C, 20 min). One hundred milliliters of the media in Erlenmeyer flasks (250 *ml*) were inoculated with 5 *ml* of spore suspension (1×10^6 spores/ml) and they were incubated at 30°C for 8 days under shaking at 120 rpm.

Physical treatment of lignocellulosic materials. The lignocellulosic materials derived from different origins were thoroughly washed, dried and ground to particles of

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1~2 mm. They were soaked in 1% NaOH, treated with hydrogen peroxide containing $MnSO_4$ for alkali treatment and autoclaved for delignification (Takagi, 1987). After the alkali treatment, the materials were washed with tap water repeatedly until being neutralized at room temperature.

Enzyme preparation. At time intervals, cultures were harvested by filtration through Millipore membrane (0.2 μm). The filtrate was centrifuged at 12,000 rpm at 4°C and the supernatants were dialyzed and used as source of crude extract for xylanase, carboxymethylcellulase (CMCase), β-xylosidiase and protease activities.

Enzyme assays. Xylanase activity was assayed using birch wood xylan (Sigma, St. Louis, MO, USA) as a substrate. A 1.0 *ml* reaction mixture contained a 0.5 *ml* of diluted enzyme solution and 0.5 *ml* of a 1% (w/v) xylan suspension in 50 mM citrate buffer, pH 5.4. The mixture was incubated at 50°C for 10 min and reducing sugar produced was determined by the dinitrosalicylic acid method (Miller, 1959) using D-xylose as the standard. One unit of enzyme activity was defined as the amount of enzyme, which releases 1 μmol xylose equivalent per min. A similar method was used for the CMCase assay, using 1% (w/v) carboxymethylcellulose (Sigma, Louis, MO, USA) as a substrate and D-glucose as the standard. Standard celluloytic assay was done at 45°C and pH 5.0 buffered with 50 mM sodium acetate. One unit of cellulose activity was defined as the amount of enzyme, which releases 1 μmol glucose equivalent per min. β-Xylosidase activity was determined by incubating 1 *ml* of 0.1% (w/v) *p*-nitrophenyl-β-D xylopyranoside with 0.1 *ml* of suitable diluted enzymes in 50 mM citrate buffer at pH 5.4 at 50°C for 30 min. One unit of enzyme activity was defined as the amount of enzyme producing 1 μmol of *p*-nitrophenol per min. Protease was assayed using 1% casein in 100 mM sodium phosphate buffer pH 7.0 as substrate. One *ml* casein was incubated at 40°C for 1 h with 1.0 *ml* of suitably incubated culture filtrate. The reaction was stopped by the addition of 2.0 *ml* of 300 mM trichloroacetic acid. After mixing on a vortex mixer, the tubes were centrifuged at 3,000 rpm for 10 min. The released tyrosine was estimated by the method of Bradford (1976) using a tyrosine standard curve. One unit of enzyme activity was defined as the amount of enzyme producing 1 nmol of tyrosine per min.

Partial purification of enzyme. Culture of *A. flavus* was prepared using basal liquid medium containing 5 g rice barn as described above. Following filtration through a Millipore membrane (0.2 μm), protein in the supernatant fluid were precipitated with $(NH_4)_2SO_4$ on ice. The precipitate was recovered by centrifugation at 20,000 rpm for 30 min, dissolved in 100 mM phosphate buffer (pH 7.2), and the dialyzed three times against 5 *l* of distilled water at 4°C overnight. The protein solution was concentrated in a rotor-evaporator (Rotovac, Switzerland) from 50 *ml* to 1 *ml*. The protein concentration was determined according to Bradford (1976).

Effect of temperature and pH. The effect of temperature on the production of xylanase by *A. flavus* was investigated at seven different temperatures for 8 days. The xylanolytic activity was determined as described above. To observe the effect of pH, the aliquots of the culture filtrates were adjusted to pH 3~12 using 50 mM buffers such as citric acid/Na_2HPO_4 for pH 3~6, $NaHPO_4$/Na_2HPO_4 for pH 6~8, Tris-HCl for pH 7~9, glycine/NaOH for pH 9~11 and $NaHCO_3$/NaOH for 11~12. Each aliquot was incubated at room temperature for 1 h and the remaining xylanase activity was assayed as previously described.

Detection of renatured xylanase isoforms after SDS-PAGE. To detect renatured xylanase isoforms, electrophoresis was performed on a 15.0% SDS-polyacrylamide gel containing 0.01% of birch wood xylan. Culture filtrate was heated for 10 min at 70°C with 2-mercaptoethanol before application to the gel. After electrophoresis, the gel was incubated for 2 h at 25°C with reciprocal shaking in 100 mM sodium acetate buffer (pH 5.2) containing 2.5% (v/v) Triton X-100, and then in the 500 mM Tris-HCl (pH 8.9) containing 0.01% (w/v) fluorescent brightener 28 at room temperature for 15 min. After removal of the brightener solution, the renatured gel was also incubated in distilled water in the dark for 2 h. The isoforms of xylanase were detected on a UV transilluminator.

Results and Discussion

Influence of lignocellulosic wastes as carbon source on induction of xylanolytic complex. To induce xylanase and β-xylosidase production by *A. flavus*, various carbon sources including xylan and xylose (Fig. 1) were tested. Among the pure carbohydrates used, only xylan and xylose induced xylanolytic enzyme production. Xylanase activity in a medium containing xylan was twofold higher than that in medium with xylose. In *Streptomyces* sp. (Flores *et al*., 1996), *Trichosporon cutaneum* (Liu *et al*., 1999), *Clostridium abosonum* (Rani and Nand, 2000) and *Thermomyces lanuginosus* (Damaso *et al*., 2000), xylan and xylose also induced the highest levels of xylanase. It means that substrates from xylanase and xylose play an important role in xylanase production.

Among the agricultural wastes examined, the fungus was able to produce highly active xylanase and β-xylosidase on rice bran, barley bran, corn cob, rice straw, and

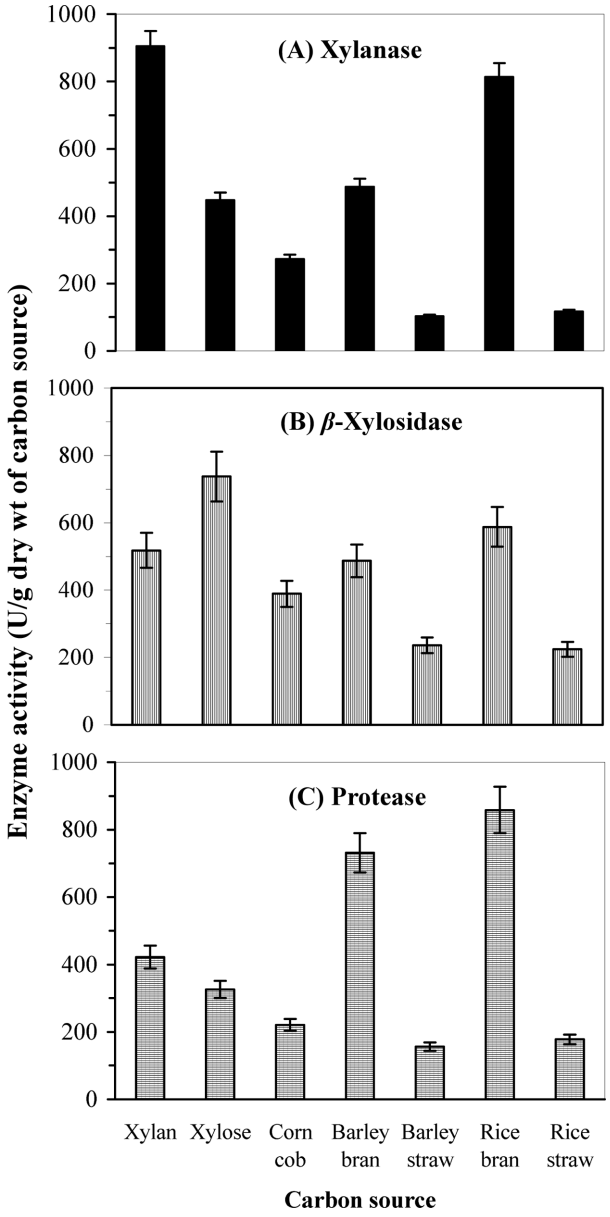

Fig. 1. Production of xylanase, β-xylosidase, and protease by *A. flavus* in basal liquid media including different lignocellulosic materials as carbon source.

barley straw as substrates. The highest value of xylanase activity was observed in rice bran medium (Fig. 1). Souza *et al.* (2001) and Ferreira *et al.* (1999) reported that xylanase production reached the highest levels when using wheat bran, corn con and sugar cane bagasse in solid cultures of *A. tamarii*, correspondingly. Moreover, wheat bran induced the high expression of xylanase in liquid cultures of *A. vesicular* (Christov *et al.*, 1999). In the culture of *A. foetidus*, *A. oryzae*, *A. niger*, and *A. phoenicis* (Christov *et al.*, 1999), however, corn cob induced the production of higher levels of xylanase activity than those induced by xylan. In the cultures of *A. flavus*, xylanase production in a medium containing barley bran or rice bran was approximately three to eight times higher than in the media containing other lignocellulosic materials. The production of xylanase and xylosidase in a medium containing rice bran was approximately 90% and 80% of that observed in xylan and xylose medium, respectively.

In this study, all the lignocellulosic materials induced very low levels of cellulolytic activity, in which CMCase activity was only 0.4 U/g dry weights of lignocellulosic materials in all of the cultures, which could confer the applicability of this enzyme complex in biotechnological processes requiring the absence of cellulose (data not shown). Protease were also found at low levels (150~220 U/g dry weights) in barley straw, rice straw and corn cob, but a high proteolytic activity (731 and 858 U/g dry weights) was observed in barley bran and rice bran cultures, respectively. These results may explained by the chemical composition of these materials. Corn cob, barley straw and rice straw contained only 2~5% of protein in their composition (Dueñas *et al*, 1995; Haddad, 2000; Madrid *et al.*, 1999; Prakash *et al.*, 1996), while barley bran and rice bran contained 11~16% of proteins (Hoebler *et al.*, 1998; Prakash *et al.*, 1996; Ryu *et al.*, 2002). Therefore, the proteolytic activity was induced to higher level by barley bran and rice bran than other lignocellulosic materials. The co-production of high proteolytic activity may be a serious problem for the maintenance of enzyme stability. Reducing proteolytic degradation during downstream process may be minimized by several strategies, including the addition of a protease inhibitor (Ferreira *et al.*, 1999). Nevertheless, these strategies cause to increase the cost of the process (Walsh and Headon, 1994).

The production profiles of xylanase, β-xylosidase and protease are exhibited in Fig. 2. The activities of xylanase and β-xylosidase reached at the highest level after 6 days incubation for all of the five different lignocellulosic materials. However, the increasing the biomass production was slower in barley and rice bran cultures than in corn cob, barley, and rice straw cultures (data not shown), possibly caused by the high protein contents in barley and rice bran (Hoebler *et al.*, 1998; Prakash *et al.*, 1996; Ryu *et al.*, 2002). In Figs. 2B and 2D, therefore, the proteolytic activity was observed at the initial incubation period in barley and rice bran cultures. The induction of protease was earlier than that of xylanolytic enzymes in barley and rice bran cultures. Similar phenomena were reported in *A. tamari* in solid-state fermentation (Ferreira *et al.*, 1999).

Effect of culture conditions on xylanase production. Fig. 3A shows that the pH profile of the supernatant xylanolytic activity of *A. flavus* K-03 with the highest level at pH 6.5~7.0. The optimum temperature of xylanolytic activity is 25~30°C (Fig. 3B). These results are in accordance with broad range pH optima for xylanase from

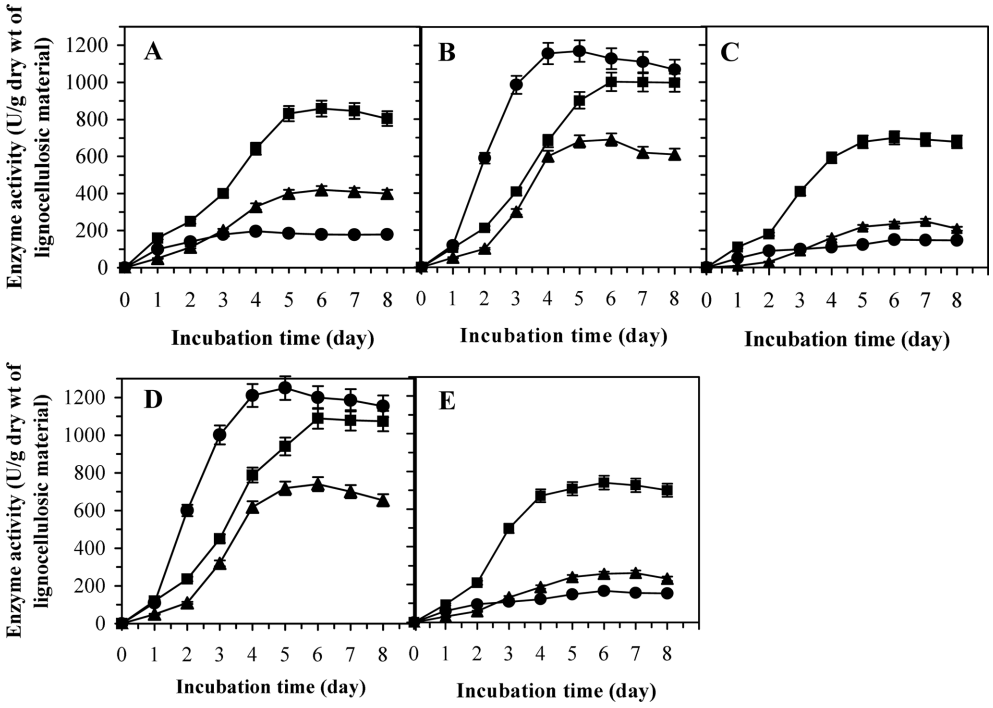

Fig. 2. Time course of xylanase (■), β-xylosidase (▲), and protease (●) production in *A. flavus*. (A), corn cob cultures; (B), barley bran cultures; (C), barley straw cultures; (D), rice bran cultures; (E), rice straw cultures.

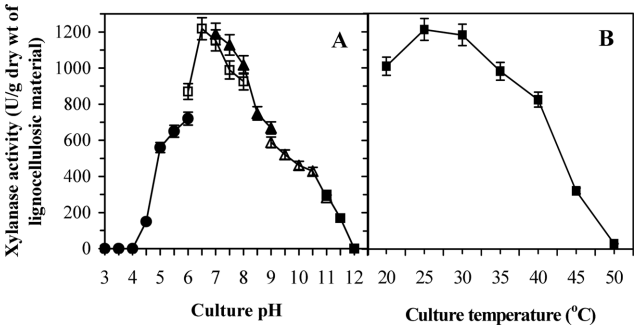

Fig. 3. Influences of culture pH (A) and temperature (B) on xylanase activity in *A. flavus* grown on rice bran. (A), Enzyme activity was determined at room temperature. (B), it was measured in 50 mM buffers such as (●), citrate buffer (pH 4~6); (□), $NaHPO_4/Na_2HPO_4$ buffer (pH 6~8); (▲), Tris-HCl buffer (pH 7~9); (△), glycine/NaOH buffer (pH 9~11); (■), $NaHCO_3$/NaOH buffer (pH 11~12).

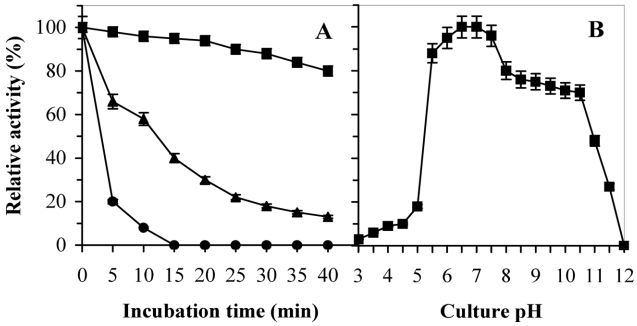

Fig. 4. Thermostability profiles (A) of xylanase from *A. flavus* strain K-03 at pH 6.5, 40°C (■), 50°C (▲) and 60°C (●), and stability of xylanase at different pH (B).

A. giganteus reported by Coelho and Carmona (2003). However, it was confused that different lineages of the genus *Aspergillus* showed different optima pHs for xylanase production. Optimal pH observed for xylanase induction by *A. nidulans* was 6.8 (Fernándes-Espinar *et al.*, 1992) and 4.0 by *A. awamori* (Smith and Wood, 1991). The growth of *A. flavus* K-03 in barley and rice bran cultures were better in neutral and alkali pH with a maximum of 8.5, which demonstrates that this fungus is able to grow in alkaline environments (Raper and Fennel, 1965).

Initial properties of the crude xylanase. The thermal stability of xylanase activity was relatively low (Fig. 4A). After 90 min, the activity was decreased to 50% of the initial activity at 40°C, and after 4 h, only 10% of activity was remained (data not shown). At 50°C, however, 13 min was enough to reduce the activity to 50%. There were markedly decreases in activities at 60°C and it took only three min to diminish xylanase activity to 50% of the initial activity. Xylanase from fungi and bacteria were often showed the optimum activity at 45~50°C, however it is usually inactivated above 60°C (Lan-Phan *et al.*, 1998). The stability against culture pHs are exhibited in Fig. 4B, the xylanolytic activity of *A. flavus* K-03 was shown at very low levels in the pH ranging from 3 to 5 and 11 to

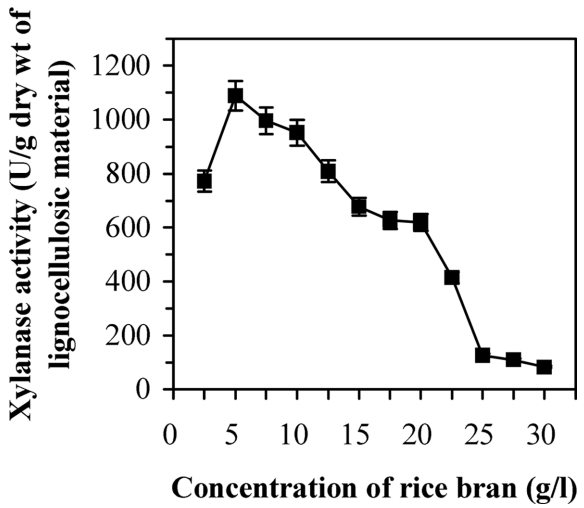

Fig. 5. Xylanase activity of *A. flavus* K-03 grown in basal liquid medium containing different concentrations of rice bran at 25°C and pH 6.5.

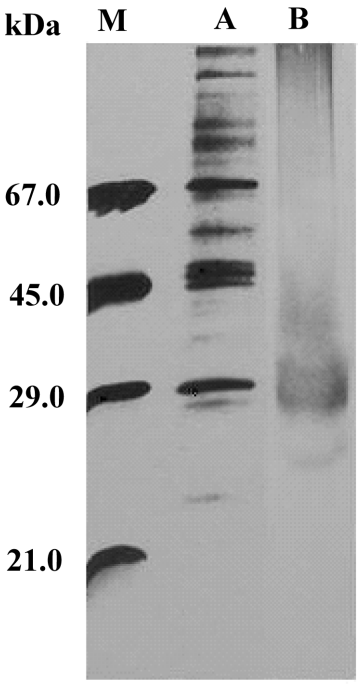

Fig. 6. SDS-PAGE analysis of the culture supernatant ultrafiltrate from *A. flavus* K-03 on the 15.0% polyacrlamide gels containing 0.01% of birch wood xylan. Soluble proteins (20 μg per lane) were stained with silver nitrate. Lanes: M, molecular size markers (bovine albumin for 67.0 kDa, egg albumin for 45.0 kDa, carbonic anhydrase for 29.0 kDa and for 21.0 kDa); A, soluble protein; B, renatured xylanase.

12, and was very stable in the pH intervals from 5.0 to 10.5. This result means that the stability of xylanase produced by *A. flavus* in an alkali condition supports the possible application in some industrial processes as a potential agent. Nevertheless, low thermal stability is a barrier for certain applications, which could be solved by further studies (Coelho and Carmona, 2003; Lemos *et al.*, 2000).

Effect of different concentrations of rice bran on xylanase production. The concentration of carbon source was verified if it could influence the xylanase activity. The basal liquid medium was supplied with different concentrations of rice bran, from 0.25% to 3.0%. As shown in Fig. 5, the production of xylanase was reached the highest level when 0.5% of rice bran was added. Xylanase production rapidly increased at low concentrations of the carbon sources, such as 0.25~0.5%, after that steady decline at higher concentrations of substrate, from 1.0% to 2.0% (Fig. 5). It suggests that decomposition products such as low molecular weight oligosaccharides existed in the rice bran contributes to the suppression of xylanase production in particular when high rice bran concentrations are tested (Lan-Phan *et al.*, 1998; Mes-Hartree *et al.*, 1998). As the accumulated hydrolysis products lead to repress enzyme production at higher substrate concentrations, the xylanase was also be possibly suppressed.

Analysis of xylanase isoforms by SDS-PAGE. Only one band of xylanase isoform was observed on SDS-polyacrylamide gel (Fig. 6). A single band of putative xylanase, which corresponded to a molecular mass near 28.5 kDa, was intensely stained. Xylanases documented in different microorganisms including alkali-tolerant *Aspergillus fischeri* (Chandra and Chandra, 1996), different alkali-tolerant or alkaliphilic bacteria have low molecular weights about 16~85 kDa (Blanko and Pastor, 1993; Wong *et al.*, 1988).

Based on the results, the present work suggests that *A. flavus* K-03 was capable of producing xylanase from natural agricultural wastes. This promise the utilization of low-cost carbon sources as a substrate and for application in biological pulp bleaching. In addition, genetic manipulation of the strain is necessary to increase the xylanase production.

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