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PURIFICATION AND CHARACTERIZATION OF A XYLANASE FROM *ASPERGILLUS NIGER* DB 106

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SUMMARY

When growing in solid state fermentation with corn cob as carbon source, strain *Aspergillus niger* DB 106 produced various xylanases. The predominant endoxylanase (signed as xylanase P) was purified to homogeneity by a four-step procedure including strong anion exchanger column, Econo Pac P6 (Bio-RAD) Gel filtration column, anion exchanger chromatography Econo Pac High Q and Sephadex G-50 gel filtration. Some physico-chemical properties of the xylanase P were characterized: The purified xylanase P showed a single protein band with a molecular mass of 32 kDa as estimated by SDS-PAGE and zymogram analysis with oat spelts xylan (OSX). It had an optimum activity at pH and temperature of 5.5 and 55°C, respectively. This enzyme was stable at a wide range pH (2.5 - 8.0), however it was not thermostable (lost activity at 60°C). V_{max} and K_m values were 20000 units.mg^{-1} and 38 mg.ml^{-1}, respectively, as determined in the enzyme reaction with insoluble OSX as substrate at 55°C and pH 5.5. The enzyme was slightly inhibited by Zn^{+2}, Mn^{+2}, Fe^{+2} and Hg^{+2}, but not by Na^{+}, K^{+}, Ca^{2+}, Cu^{2+}, Co^{2+}, Mg^{2+}, and EDTA. The xylanase P could not degrade carboxylmethyl cellulose (CMC), p-nitrophenyl galactopyranoside and p-nitrophenyl xylopyranoside. All these data showed that xylanase P was a true xylanase, with no cellulase, β-galactosidase, amylase and beta-xylodase activities. Hydrolysis products of the purified enzyme action on OSX substrate were analyzed on TLC showing that xylanase P was an endoxylanase that completely degraded xylan to mainly short xylooligosaccharides and minor amount of xylose.

Keywords: *Aspergillus niger DB 106, chromatography, kinetics, purification, xylan, xylanase*

INTRODUCTION

Xylan is the main component of hemicellulose and is composed of β-1,4-linked xylopyranose chains with 4-O-methyl-D-glucuronic acid, arabinose, O-acetyl, and uronic acids subtituents. Due to its heterogeneity and complexity, the complete hydrolysis of xylan requires a large variety of cooperatively acting enzymes including endo-β-1,4-xylanases (EC 3.2.1.8), β-xylosidase (EC 3.2.1.37), enzymes which cleave side chain sugars from the xylan backbone, such as α-arabinofuranosidases (EC 3.2.1.55) and acetyl esterases (EC 3.1.1.6), among which endo-β-1,4-xylanases play a key role, which hydrolyze the xylan backbone at non modified residues producing short xylo-oligosaccharides (Levasseur *et al.*, 2005). Indeed, complete xylanolytic enzyme systems, including all of these activities, have been found to be quite widely spread among fungi, actinomycetes and bacteria, and some of the most important xylanolytic enzyme producers include the *Aspergilli*, *Trichodermi*, *Streptomycetes, Phanerochaetes, Chytridiomycetes, Ruminococci, Fibrobacteres, Clostridia and Bacilli* (Collins *et al.*, 2005). *Aspergillus* genus could produce many industrial enzymes including xylanases. *Aspergillus niger* produces both acidic and alkaline xylanases, and some of them were purified to homogeneity and characterized using soluble and insoluble xylan (Kulkarni *et al.*, 1999).

From the biotechnological standpoint, xylanases have wide applications in production of ethanol, aroma, fruit juices, baking, textile, paper and pulp industries, and recently were extensively used in animal husbandry as growth promoter (Wu, Ravindran, 2004; Danicke *et al.*, 1999).

There are many reports about xylanases including its purification and characterization (Subramaniyan, Prema, 2002; Silva *et al.*, 1999). However, in Vietnam, to date there were few reports about o xylanases (Phuong Phu Cong, Mai Thi Hang, 2005; Dao Thi Hai Ly, Mai Thi Hang, 2007). Phuong Phu Cong and Mai Thi Hang (2005) reported that *A. niger* GM56 isolated from mangrove mud and its derived

mutant *A. niger* DB 106 could produce high amount of xylanases on agriculture by- product. The specific activities of *A. niger* DB 106 xylanolytic enzymes were high during growth on xylan, or lignocellulosic residues. The crude xylanase was very active and stable at acidic pH 2.0 - 2.5 and was promised candidate for producing xylanase used for pig and poultry. The *A. niger* DB 106 crude enzyme preparation showed very good effect on chicken growth (Tran Huu Phong *et al.*, 2007).

In order to have good understanding of this xylanase for its application, especially in animal husbandry, this study attempted to purify the major endoxylanse of this filamentous fungal strain to elucidating its properties.

MATERIALS AND METHODS

Microorganism and culture medium

Strain *Aspergillus niger* DB 106 (mutant variant of wild strain *A. niger* GM56 treated by UV rays) from Microorganism Culture Collection of Micro-Biotechnological Lab, Hanoi National University of Education.

The optimum medium for *A. niger* DB 106 xylanase production contained 30% ground corn moisturized to 70% with mineral solution consisting of (g/l): KH_2PO_4, 4.2; KCl, 0.5; KNO_3, 0.5; $MgSO_4$, 0.5 ; $FeSO_4$, 0.01; NaCl, 1.25. (Phuong Phu Cong, Mai Thi Hang, 2005).

Methods

Activity assays

Xylanase activity was determined by the method of Bailey *et al.* (1992). One unit of xylanase was defined as the amount of enzyme required to release 1 µmol D-xylose from Oat spelt xylan (Sigma) per min at experiment condition. Reducing sugar was determined by dinitro salysilic acid method (Miller, 1959) using xylose as standard sugar.

Protein assays

Protein concentration was estimated by Bradford's method (1976) and at 280 nm by Warburg and Christian (1942) with bovine serum albumin (BSA, Sigma) as a standard.

Xylanase purification

Xylanase purification was carried out thought 4 steps procedures The culture filtrate of *A. nige* DB106 was concentrated by using AMICO ultrafiltration system with 10 kDa cut-off membran (PM10, Milipore) and it was used as crude enzym for further purifications as described bellows.

Step 1: *Strong Anion Exchanger column* (Bio Rad). Crude enzyme was applied on anion exchange column equilibrated by 50 mM phosphate buffer pH 6.5, on FPLC system. Proteins bound on colum were eluted by gradient 0 - 1 M NaCl in 50 mM phosphate buffer, pH 6.5. Fractions with xylanas activities were collected.

Step 2: *Econo Pac P6* (Bio-Rad). Enzym solution collected was dialyzed and applied o Econo Pac P6 (Bio-Rad) and eluted by 50 mM phosphate buffer, pH 6.5 with rate 30 $ml.min^{-1}$ Fractions with xylanase activities were collected.

Step 3: *Econo Pac High Q* (Bio-Rad). Enzym solution collected in step 2 was concentrated an applied on Econo Pac High Q column. The sampl was eluted by gradient 0 - 1 M NaCl in 50 mM phosphate buffer pH 6.5. Fractions with xylanas activity was collected and dialyzed in 50 mM phosphate buffer pH 6.5.

Step 4: *Sephadex G-50 chromatography* (Sigma) *The* enzyme solution was applied on Sephadex G-5 column and eluted by 50 mM phosphate buffer, pH 6.5 with rate of 18 $ml.min^{-1}$. Xylanase activit fractions were collected and concentrated.

Molecular mass estimation and zymogram analysis

SDS-PAGE was carried out according to the method of Laemmli (1970) with 12.5% polyacrylamide gel and using standard protein rang 12 - 78 kDa (Protein Standard Mixture IV include Cytochrom C-12300 Da; Myoglobin-17200 Da; Carbonic Anhydrase-30000 Da; Ovalbumin-45000 Da; Albumin-66250 Da; Ovotransferrin-76000 - 78000 Da, Sigma). Protein bands were stained by Coomassie Blue R250.

Zymogram analysis was carried out according to the method of Schwarz (Shah, Madamwar, 2005). The gel was added 0.1% OSX before adding SDS and TEMED. After electrophoresis SDS was removed from gel by immersing in 2.5% Triton X-100 three times (15 min per each time). The gel was incubated in 100 mM sodium acetate pH 5.5 at 40°C in 5 h and, then immersed in 0.1% in red Congo 10 min and band with xylanase activity was appeared

by rinsing the gel in 1 M NaCl. Then gel was incubated in 5% acetic acid for preservation.

Determination of temperature, pH and metal ion effect

Effect of pH: Enzyme-substrate reaction was carried out on experimental condition with range of pH 2.5 - 8.0 at intervals of 0.5 with three buffers: Glycine-HCl (pH 2.5 - 3.5); sodium acetate (pH 3.5 - 5.5), and Sorensen (pH 5.5 - 8.0). pH stability of enzyme was estimated by incubating purified enzyme in buffers with pH range 2.5 - 8.0 at 4°C for 120 min.

Effect of temperature: Enzyme-substrate reaction was carried out at different temperatures (35 - 70°C). Temperature stability was estimated by incubating purified enzyme at various temperatures (15 - 80°C).

Effect of metal ion and EDTA: purified enzyme was incubated in different solutions containing 1 mM metal ions including Na^+, K^+, Ca^{2+}, Cu^{2+}, Co^{2+}, Mg^{2+}, Zn^{2+}, Mn^{2+}, Fe^{2+}, Hg^{2+}, and EDTA. The residual activities were determined as stated.

Analysis of hydrolyzed products

Hydrolyzed products of OSX by purified xylanase were analyzed by TLC. Purified enzyme-substrate mixture was incubated at 40°C and the sample was centrifuged at 10,000 rpm in 5 minutes. The supernatants were used for TLC analysis in a running solvent system containing n-butanol: pyridine: H_2O (6:4:3, v/v). TLC was dried and sprayed with solution containing diphenylamin: 2 g; aniline: 2 ml; acetone: 100 ml; 15 ml 80% phosphoric acid. D-xylose was used as standard.

Determination of V_{max} and K_m

The kinetic constants K_m and V_{max} were estimated following the method of Lineweaver and Bruk, using OSX as the substrat (Lineweaver, Bruk, 1934).

RESULTS AND DISCUSSION

Enzyme purification Zymogram analysis showed that strain *A. niger* DB 106 produced various xylanases of various MW (Figure 2-I) with a predominant endoxylanase (hereafter signed as xylanase P). Results on xylanase P purification were described in table 1 and figure 1.

Step 1 (*Strong Anion Exchanger*): Various proteins peaks were obtained (Figure 1). High endoxylanase activity was observed at 0.3 M NaCl, also at 0.45 - 0.5 M NaCl but lower endoxylanase activity (Figure 1-I).

Step 2 (*Econo Pac P6*): Two protein peaks were found on chromatograph (Fig 1-II, one of them possessed endoxylanase activity. At this stage, no cellulase, amylase, protease activity were detected, meaning that those enzymes in crude enzyme extract were eliminated (data not shown).

Step 3 (*Econo Pac High Q*): Two protein peaks were observed (Fig.1-III) and endoxylanase activity was found at peak corresonding to the peak with 0.2 - 0.3 M NaCl elution

Step 4 (*Sephadex G-50*): Three protein peaks were observed (Fig. 1-IV) and peak 2 showed endoxylanase activity.

Molecular mass and zymogram analysis

Xylanase P was purified by a four-step chromatographic procedures. The purified enzyme showed a single protein band with molecular mass of about 32 kDa on SDS-PAGE.

Zymogram analysis results showed that one active band was found corresponding to the band on SDS-PAGE (Figure 2-II). Thus, using classical chromatography methods the xylanase P was purified to homogeneity from crude enzymes with 18 folds with a specific activity of 731 IU/mg and a yield of 4%. There were several xylanases from different origins were reported to be purified using various classical chromatography techniques as well (Kluepfel *et al.*, 1990; Ghosh, Nanda, 1994; Lin *et al*, 1999; Silva, 1999; Carmona *et al.*, 2005). A xylanase II from *Aspergillus fumigatus* Fresenius with molecular mass of 19 kDa was purified to homogeneity by using five purification steps (Silva *et al.*, 1999) and *Aspergillus versicolor* xylanase II with molecular mass of 32 kDa was purified with 4 - steps procedures using chromatography techniques (Carmona *et al.*, 2005). The new methods of efficient purification of xylanase using Eudragit were also reported (Sarda *et al.*, 2000). The separation of xylanases from the crude culture filtrates of *Aspergillus* sp. 5 and *Aspergillus* sp. 44 was carried out using affinity precipitation with a commercially available enteric polymer Eudragit S100 (Gawande, Kamat, 1999; Sarda *et al.*, 2000) with a yield of 85.3 and 82.7%, respectively. However, application of this method was not successful with *A. niger* DB 106 xylanase. We have successfully purified *A. niger*

xylanase P to homogeneity using several very simple classical techniques as stated above. The ease of purification procedure is very useful in producing xylanase P for analytical purpose. Moreover, for industrial application especially in paper and pulp industry, the partially purified xylanase P free of cellulase, protease, and other hydrolytic enzyme might be easily obtained using only two purification steps of strong ion exchange and gel filtration (Econo Pac P6), the latter could be replaced with Cephadex G50 gel filtration with the same effect (data not shown). Xylanase applied in animal feed need not to be purified, the enzyme could be applied in almost crude form.

Table 1. Purification endoxylanase P from *A. niger* DB 106.

Steps	Volume (ml)	Total protein (mg)	Total units (IU)	SA (IU/mg)	Fold	Yield (%)
Crude	1000	856.7	34713	40.5	1	100
Strong Anion Exchanger	100	62.8	13682	217.7	5.4	39
Econo Pac P6	100	45.5	10694	235.1	5.8	31
Econo Pac High Q	100	18.7	5497	294.4	7.3	16
Sephadex G-50	100	2.1	1534	730.5	18.0	4

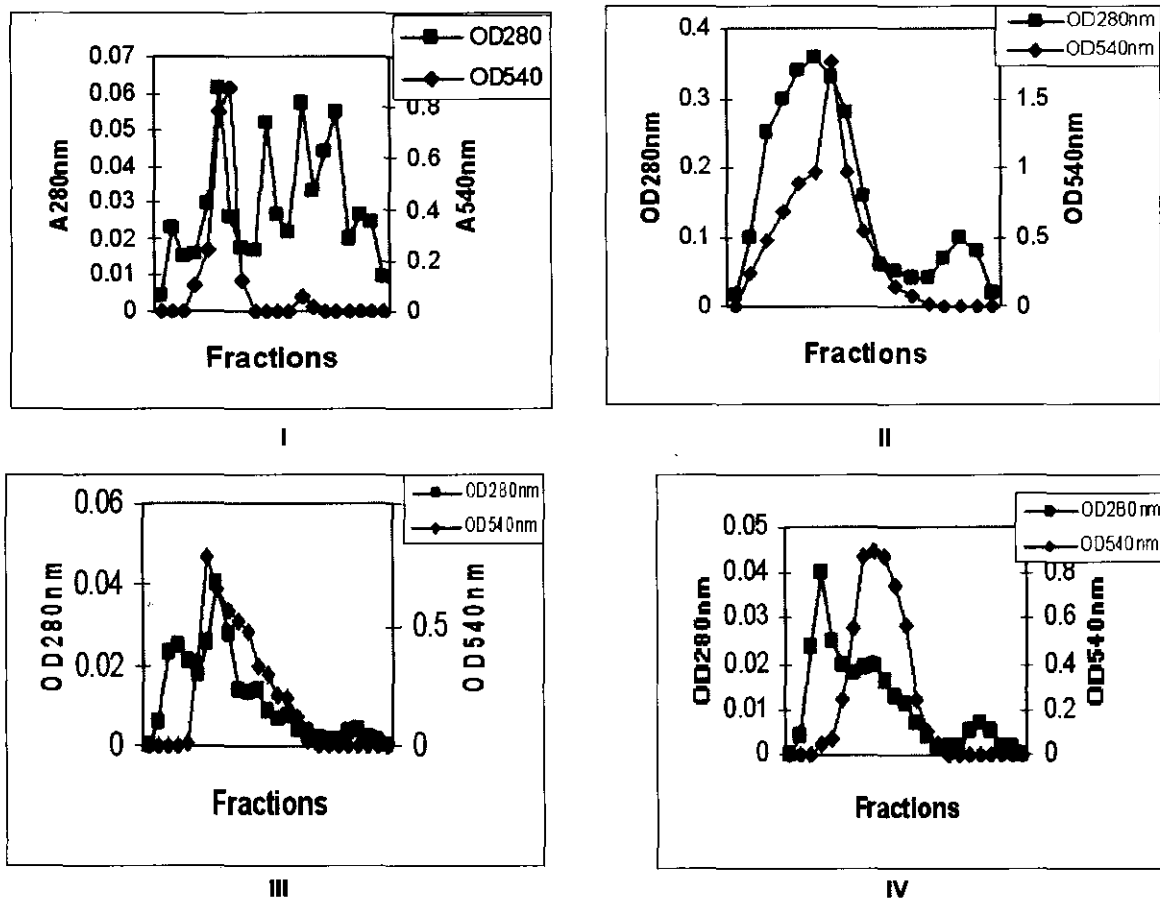

Figure.1 Strong Anion Exchanger chromatography (**I**); Step 2 (Econo Pac P6 Gel filtration (**II**); Econo Pac High Q chromatography (**III**). Sephadex G-50 Gel filtration (**IV**). Note: OD_{280nm}: protein concentration, OD_{540nm}: xylanase activity.

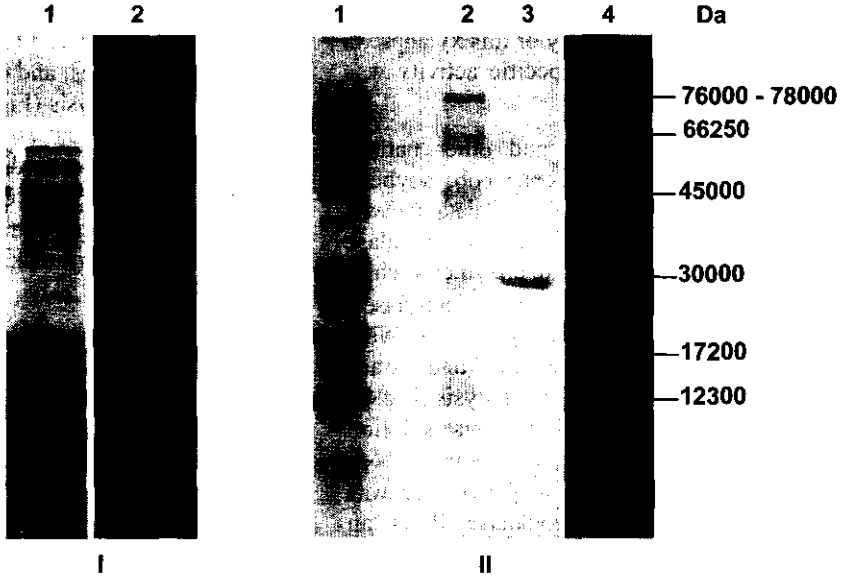

Figure 2. SDS-PAGE of the xylanase from *A. niger* DB106. (I) 1. Crude extract from *A. niger* DB 106; 2. Multi xylanase by zymogram analysis; (II) 1. Crude extract from *A. niger* DB 106; 2. Protein Standard Mixture IV; 3. Pury xylanase P; 4. Xylanase P activity by zymogram analysis.

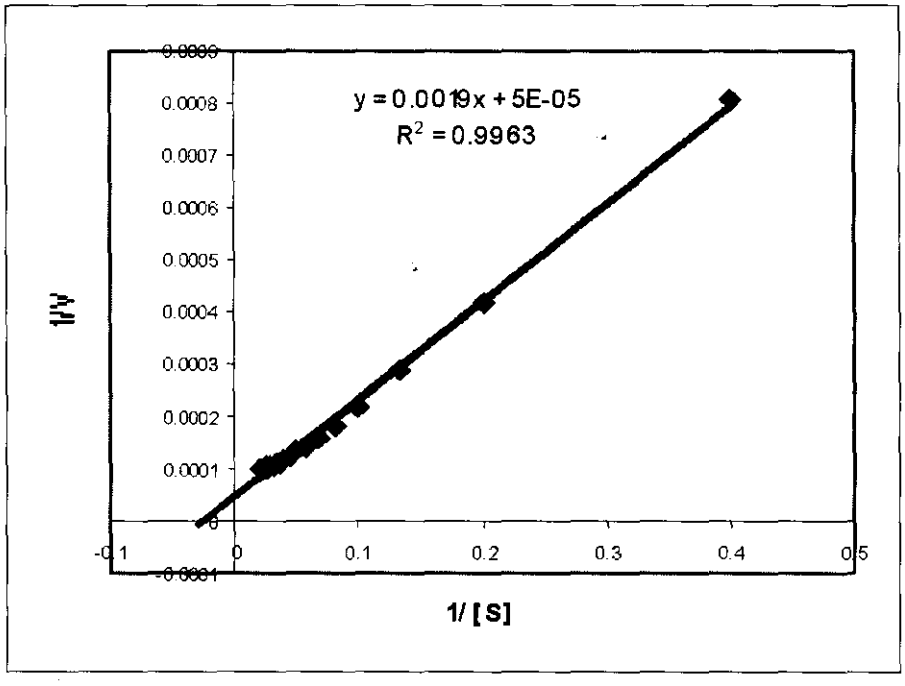

Figure 3. Lineweaver-Burk plot.

Determination of V_{max} and K_m values

V_{max} and K_m values estimated by using Lineweaver-Burk method on insoluble OSX at 55°C and pH 5.5 was 20,000 IU.mg^{-1} and 38 mg.ml^{-1} respectively (Figure 3)

K_m of fungal xylanases reported so far were from 0.58 to 40 mg.ml^{-1} and V_{max} were in range of

0.16 to 100,000 $mg.ml^{-1}$ (Subramaniyan, Prema, 2002). As can be seen the affinity of this xylanase is low (K_m is high), however its specific activity was high (20,000 $IU.mg^{-1}$).

It could digest pure OSX and other natural xylans contained in rice bran, corn cob, soybean meal, rice hull (data not shown). These data showed that xylanase P is true xylanase, free of cellulase activity and several other hydrolytic enzymes such as β-galatosidase, beta-xylodase and amylases. Products of hydrolyzed OSX by xylanase P were mainly short oligo-xylosaccharides, and trace amount of xylose as shown on TLC analysis (Fig. 4-I), while under the action of crude xylanase solution beside oligo-xylosaccharides, it could be clearly seen high amount xylose formed due to β-xylodase was presented. This demonstrates xylanase P is an endoxylanase, which breaks xylan chains in random fashion.

Substrate specificity of xylanase P

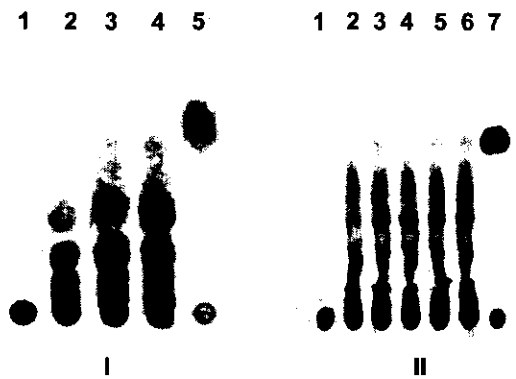

Figure 4. I. TLC analysis of hydrolyzed products on OSX by purified xylanase P; 1. Oat speltm xylan; 2 – 4. Hydrolyzed products after incubated 15, 30, 60 min; 5-D-xylose. **II.** TLC analysis hydrolyzed products on OSX by crude xylanase; 1. 1% xylan; 2 – 6. Hydrolyzed products after incubation 15, 30, 60, 90 min; 7. D-xylose.

Purified xylanase P could not degrade carboxylmethyl cellulose (CMC), p-nitrophenyl galactopyranoside, p-nitrophenyl xylopyranoside. It could digest pure OSX and other natural xylans contained in rice bran, corn cob, soybean meal, rice hull (data not shown). These data showed that xylanase P is true xylanase, free of cellulase activity and several other hydrolytic enzymes such as β-galatosidase, beta-xylodase and amylases. Products of hydrolyzed OSX by xylanase P were mainly short oligo-xylosaccharides, and trace amount of xylose as shown on TLC analysis (Figure 4-I), while under the action of crude xylanase solution beside oligo-xylosaccharides, it could be clearly seen high amount xylose formed due to β-xylodase was presented. This demonstrates xylanase P is an endoxylanase, which breaks xylan chains in random fashion.

Effect of temperature, pH and metal ion

Effect of temperature: Xylanase P could react well in temperature range of 35 - 70°C with optimum temperature at 55°C (Figure 5-II). Enzyme stability under temperatureat 50°C, 55°C and 60°C (Figure 5-I) at different times were investigated and the results showed that purified xylanase P was less stable than it was in crude solution (Tran Huu Phong *et al.*, 2007). But at 50°C, the stability of xylanase P was higher than that of crude solution.

Effect of pH: As showed in figure 6, xylanse P was active at a wide pH range (2.5 - 7.0) with highest activity at pH 5.5 - 6.0. At alkaline pH, the activity was low and lost activity at pH 8.0. This enzyme was stable at pH range of 4.5-8.0 and the most stable at pH 6.0 - 6.5. The enzyme retained activity of 35% when it was incubated at pH 2.5 in 120 min. This specific property ensures the activity of this xylanase in animal digested systems, where pH of stomach was from 2.5 - 4.0.

Table 2. Effect of metal ions on activity of xylanase P.

Metal ions (1 mM)	Activity remaining (%)
Na^+	103
K^+	100
Ca^{2+}	106
Cu^{2+}	104
Co^{2+}	110
EDTA	107
Mg^{2+}	100
Zn^{2+}	94
Mn^{2+}	92
Fe^{2+}	92
Hg^{2+}	88

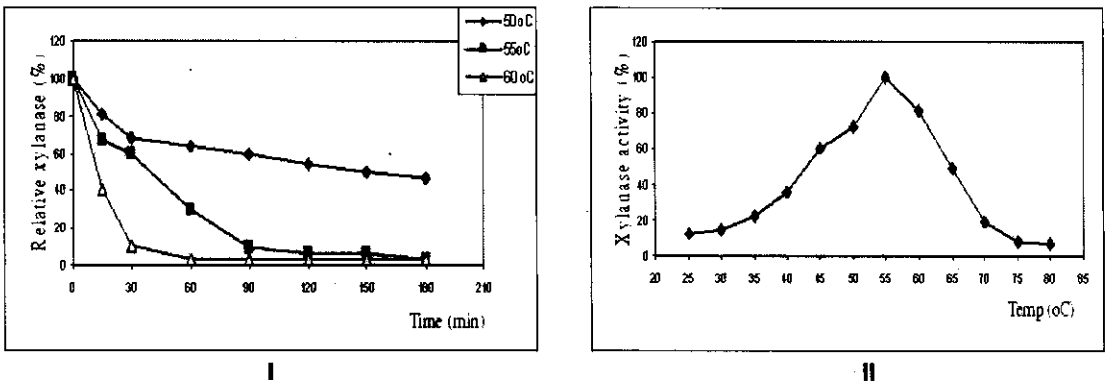

Figure 5. Temperature stability (I) and activity (II) of xylanase P.

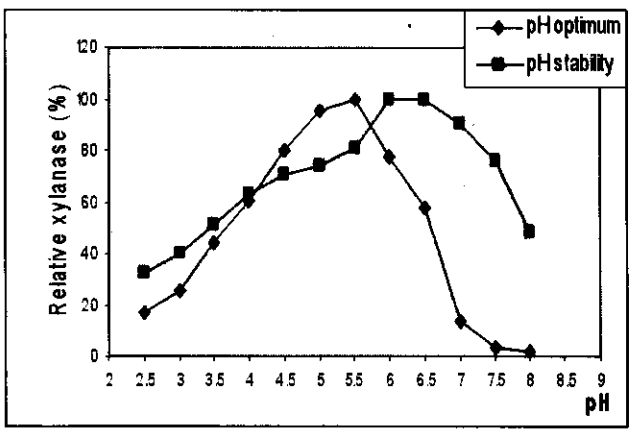

Figure 6. pH activity and stable of xylanase P.

Effect of metal ions: Effect of metal ions on activity of xylanase P is different. Hg^{+2} inhibited strongly xylanase activity, while Zn^{2+}, Mn^{2+}, Fe^{2+} showed slightly inhibition. Presence of Na^{+}, K^{+}, Ca^{2+}, Cu^{2+}, Co^{2+}, Mg^{2+} and EDTA increased activity of xylanase P (Table 2).

CONCLUSION

Xylanase P (a predominant endoxylanase in *A. niger* DB 106 crude enzyme extract) was purified to homogeneity by using some chromatography techniques with specific activity of 730.5 $IU.mg^{-1}$ protein, purification folds of 18 and yield of 4%.

Purified xylanase was about 32 kDa as estimated by SDS-PAGE, and considered to be an endoxylanase which breaks xylan chains in random fashion forming mainly short xylooligosacharides and minor amount of xylose. The properties of the purified xylanase were almost the same with that was in crude extract except the hydrolyzed products of crude extract contained higher amount of xylose due to presence of β-xylodase. It was more active and stable at acidic pH than alkaline, and was not thermostable. These data indicating that xylanase P is suitable for used as enzyme feed used in animal husbandry; however it could not be formulated in pellet form where the processing was at high temperature.

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TINH SẠCH VÀ NGHIÊN CỨU MỘT SỐ ĐẶC ĐIỂM CỦA XYLANASE TỪ CHỦNG *ASPERGILLUS NIGER* ĐB 106

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Trường Đại học Sư phạm Hà Nội

TÓM TẮT

Chủng *Aspergillus niger* ĐB 106 khi lên men bề mặt trên môi trường rắn với nguồn carbon là lõi ngô tạo ra một tổ hợp xylanase trong đó endoxylanase (kí hiệu là xylanase P) chiếm ưu thế. Xylanase P đã được tinh sạch đến đồng nhất từ tổ hợp này qua 4 bước sắc ký trên hệ thống FPLC (sắc ký trao đổi ion, sắc ký lọc gel). Một số đặc tính lý hóa của xylanase P đã được nghiên cứu: Khối lượng phân tử của xylanase P ước tính khoảng 32 kDa khi tiến hành điện di SDS-PAGE. Xylanase P hoạt động tối ưu ở 55°C và pH 5,5. Nó bền ở dải pH khá rộng (2,5 - 8,0), nhưng khả năng bền nhiệt lại không cao, mất hoạt tính ở 60°C trong 2 h. V_{max} và K_m của xylanase P tương ứng là 20.000 IU.mg^{-1} và 38 mg.ml^{-1} trên cơ chất oat spelt xylan (OSX) không tan. Enzyme này bị ức chế nhẹ bởi các ion Zn^{+2}, Mn^{+2}, Fe^{+2}, Hg^{+2}, nhưng không bị ức chế bởi Na^{+}, K^{+}, Ca^{2+}, Cu^{2+}, Co^{2+}, Mg^{2+} và EDTA. Xylanase P không phân giải carboxylmethylcellulose (CMC), p-nitrophenyl galactopyranoside, p-nitrophenyl xylopyranoside và tinh bột ngô. Tất cả các dữ liệu trên cho thấy rằng đây là loại xylanase không có hoạt tính cellulase và các enzyme thủy phân khác như α-galactosidase, β-xylodase và amylase. Phân tích sản phẩm thủy phân bằng sắc ký bản mỏng cho thấy rằng xylanase P là một endoxylanase có thể phân giải xylan theo cơ chế ngẫu nhiên tạo thành đường xylooligosaccharide chuỗi ngắn là chính và một lượng nhỏ xylose.

Từ khóa: *Aspergillus niger ĐB 106, động học, sắc ký, tinh sạch, xylanase, xylan*

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