

## PURIFICATION AND CHARACTERATION OF XYLANASE FROM *ASPERGILLUS ORYZAE* VTCC F187

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### SUMMARY

Xylanase is produced by many bacteria and fungi, among which *Aspergillus oryzae* is considered as a potential source. In this study, a xylanase was isolated and purified from the crude culture filtrate of *Aspergillus oryzae* VTCC F187 after 7 days of growth on the optimal culture containing 7% corncob and 5% soybean powder under liquid-state fermentation. After two steps purification process including gel filtration chromatography (Sephadex G-75) incorporating with anion-exchange chromatography (DEAE-sephadex), obtained xylanase was purified with the yield and purity of 24.9% and 3.91 fold, respectively. The molecular mass of the purified xylanase determined by SDS-PAGE was 32 kDa with a specific activity of 1268.0 U/mg towards 1% (w/v) of birch wood xylan. The xylanase displayed its optimum activity at 60°C, pH 6.5, and the enzyme remained active effectively within pH 3.0–5.0 and at the temperature below 37°C. Some substances were tested at concentration of 2% (v/v) such as  $\beta$ -mercaptoethanol, DMSO, Tween 80 and 10 mM NaN<sub>3</sub> slightly decreased xylanase activity and reached over 85%. While EDTA 10 mM and SDS at concentration of 2% inhibited more strongly, xylanase activity was only 77.6% and 56.6% comparing with control one, respectively. The biochemical characteristics suggested that the xylanase has a potential application, including use as a feed enzyme or using hydrolysis to produce environmentally friendly Bio-products.

**Keywords:** *Aspergillus oryzae* VTCC F187, DEAE, sephadex, xylanase

### INTRODUCTION

Xylanase or endo-1,4-beta-xylanase (EC 3.2.1.8) is a group of enzyme which plays a crucial role in the xylan hydrolysis, the most common natural hemicellulose, into short oligosaccharides and xylose (He *et al.*, 2015), (Li *et al.*, 2018).

In recent years, with its hydrolytic properties, xylanase has been used in numerous industries. Not only widely be used in paper

industries, animal feed production, fuel alcohol production, ... the use of xylanase in hydrolyzing arabinoxylan is being considered and applied in functional food production. Xylanase also is an effective tool in synthesis of xylose, a functional sugar, which is increasingly more important in pharmaceutical applications, including the replacement for glucose in diets for people with diabetes, an inhibitor to prevent the increase of glucose and insulin in blood after eating (Bae *et al.*, 2011).

Many types of xylanase productions have been commercialized on the world market. This is the reason why scientists are interested in researching synthetic sources, superior properties, methods to increase the yield and purification of xylanase. Among the xylanase-producing organisms, *Aspergillus* spp. (*A. nidulans*, *A. ochraceus*, *A. fumigatus*, *A. oryzae*, *A. niger*, *A. awamori*) are of the factors with the highest xylanase biosynthesis. Xylanases from *Aspergillus* spp. have some common characteristics, such as being effective at temperatures of 35–50°C, pH 3.0–5.5, and stable at pH ranges from 4.0 to 8.0. Along with that is the disadvantage of poor heat resistance, inactivated above 70°C. In particular, the species *A. oryzae* is a potential target of scientific researches around the world (Chipeta *et al.*, 2005), (Golubev *et al.*, 1993), (He *et al.*, 2015), (Kimura *et al.*, 2000). Besides the advantages of production of xylanase with high activity, xylanase produced from *A. oryzae* has some outstanding features such as specific structural regions which we can use mutation techniques, recombinant proteins, the catalyst... to significantly increases catalytic efficiency and heat resistance (Gao *et al.*, 2012), (Li *et al.*, 2018), Li *et al.*, 2017).

In our previous studies, we selected and cultured a number of fungal strains of *Aspergillus* to produce xylanase and evaluated the physicochemical properties of purified xylanase. The result is that we selected some strains, such as *A. awamori* VTCC F312, *A. oryzae* DMS1863, *A. niger* VTCC F017, *A. oryzae* VTCC F187 with high xylanase production (Do, Quyen 2010), (Do *et al.*, 2012), (Do *et al.*, 2009). Purified xylanase from *A. awamori* VTCC F312 has a purification of 5.3 times and 32 kDa (Do *et al.*, 2012). The team also conducted some research to optimize the culture conditions of some effective xylanase production strains such as *A. niger* VTCC F017, *A. niger* DMS1957, *A. oryzae* VTCC F187, *A. oryzae* DMS1863 (Do, Quyen 2010), (Do *et al.*, 2009). In this study, we initially purified xylanase and evaluated the physicochemical

characterization of purified xylanase from *A. oryzae* VTCC F187.

## MATERIALS AND METHODS

### Chemicals

Chemical substances used in the experiments: xylan from birch wood (Biochemika), peptone, Triton X100 (Merck), sephadex G75 (Merck), DEAE-sepharose (Heidelberg), 3,5- dinitrosalicylic acid (DNS) (Fluka),  $\text{KH}_2\text{PO}_4$ ,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $\text{NaNO}_3$ , KCl, Tween 80, and Tween 20 (BioBasic, Canada), birch wood xylan was from Biochemika (Sigma).

### Organism and growth conditions

*Aspergillus oryzae* VTCC F187 was activated on Czapek agar medium. The plates were incubated at 30°C for 3 days for spore production and then stored at 4°C to use.

### Culture conditions

Liquid-state fermentation was carried out using corncob and soybean powder as the substrates. The initial pH of the medium was adjusted to 7.0. Fermentation was carried out at 30°C for 168 hours, at 180 rpm. To obtain crude enzyme extract, the liquid mixture was centrifuged at 10000 rpm for 10 min, and the supernatant was collected and stored at 4°C prior to use.

### Determination of total soluble proteins

Protein amount was quantified based on the Bradford method with Bradford working solution and the bovine serum albumin as standard. The absorbance was measured at 595 nm (Bradford, 1976).

### Determination of xylanase activity

Xylanase activity is determined by Miller's spectrometric method (1959) (Miller, 1959) with 0.5% xylan substrate in 20 mM phosphate buffer at pH 6.5. The content of reducing sugars released in the reagent solution at the temperature of 50°C for 5 minutes is determined

by spectrophotography at the wavelength of 540 nm. The absorption is compared with the standard line of xylose sugar to work out the equivalent content of reducing sugars. One unit of xylanase activity is defined as the amount of hydrolyzed catalytic enzyme required to release 1  $\mu$ mol xylose per minute under experiment condition.

### **Purification of xylanase**

All purification steps were carried out at 4°C. The crude enzyme extract was purified through gel filtration Sephadex G75 column (2.6×26 cm) equilibrated with 50 mM potassium phosphate buffer, pH 7.5. Proteins were eluted using the same buffer at a flow rate of 25 ml/h. Fractions of 1.5 ml were collected, and those showing the highest xylanase activity were pooled. The protein in each sample was determined by taking absorbance at 595 nm. Fractions containing xylanase activity were pooled, concentrated, and loaded on to an anion exchange column (DEAE-sephadex, 2.6×26 cm) equilibrated with Tris-HCl of 50 mM (pH 8.0) buffer. Unbound proteins were eluted in the equilibrating buffer, while bound proteins were eluted with a linear gradient of NaCl (0.2–1 M) in the same buffer. The flow rate was adjusted to 25 mL/h, and the fractions of 1.5 mL were collected. Fractions showing xylanase activity were pooled and stored at 4°C for further studies.

### **SDS-PAGE**

Protein purity was examined using SDS polyacrylamide gel electrophoresis (SDS-PAGE) according to the Laemmli method (He, 2011). The gel was dyed with Coomassie brilliant blue R-250.

### **Time reaction optimum**

Time reaction optimum of the xylanase was determined by measuring the activity, using a 20 mM potassium phosphate buffer pH 6.5, at 50°C, in the time range of 5–50 minutes

### **Temperature and pH optimum**

The pH and temperature optimum of the

xylanase was determined by measuring the activity, using a 20 mM potassium phosphate buffer (pH 3.0–8.0) at 50°C, in the temperature range of 25–70°C at a pH of 6.5, respectively.

### **Temperature and pH stability**

For the determination of the temperature and pH stability, purified xylanase was preincubated at different temperatures 25, 37, 40, 50, and 60°C for 0-6 hours in 20 mM potassium phosphate buffer pH 6.5 and pH range 3.0–8.0 (20 mM potassium phosphate buffer) at 25°C for 1–6 hours, respectively. The residual activity was then determined at 60°C and pH 6.5.

### **Effect of inhibitors**

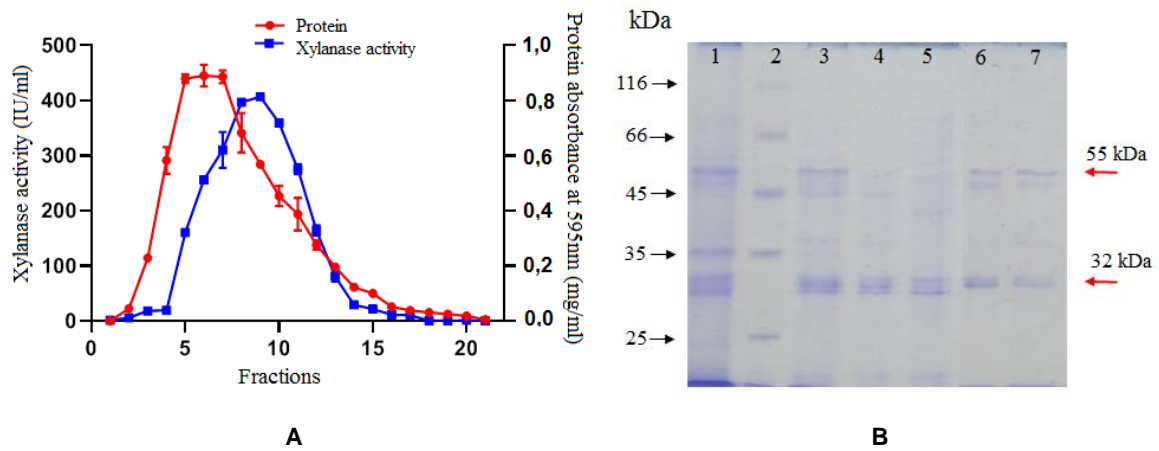
The enzyme activity of the purified xylanase was determined in the presence of 10 mM of EDTA, NaN<sub>3</sub> (sodium azide), and 2% (w/v) of DMSO (dimethyl sulfoxide), SDS,  $\beta$ -mercaptoethanol, and Tween 80.

All measurements were carried out in triplicate with the resulting values being the mean of the data obtained.

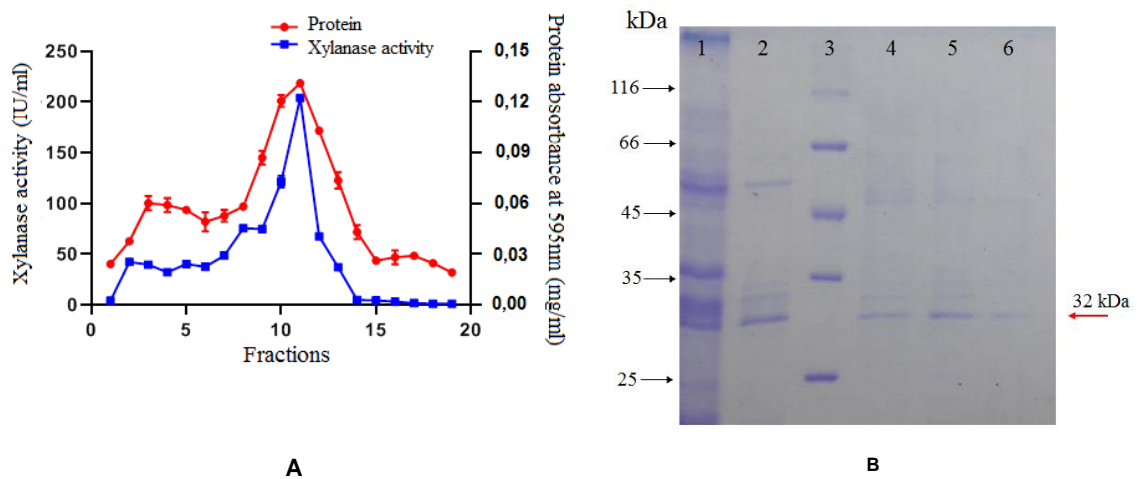
## **RESULTS AND DISCUSSION**

### **Purification of xylanase from *A. oryzae* VTCC F187**

Following 7-day solid fermentation, *A. oryzae* VTCC F187 exhibited the highest xylanase activity at 1286.0 IU/mg. The crude enzymatic extract was concentrated through a sephadex column (Fig.1A), which enhanced the purification efficacy on the DEAE-sephadex anion-exchange column (Fig. 2B). Collected fractions were assayed using xylanase activity assay test. The fractions showing enzymatic activities were subjected to SDS-PAGE to determine purity (Fig. 2B). Two-step purification was able to isolate *A. oryzae* VTCC F187 xylanase, with molecular weights of 32 kDa (Fig. 2B). The purification yield based on the enzyme activity was 24.9%, and the purification fold was 3.91 (Table 1).



**Figure 1.** Sephadex G75 performance chromatography; SDS-PAGE of xylanase purified by sephadex G75 (B) (Lane 1: crude supernatant; 2: molecular weight markers; 3-7: gel filtration fractions).



**Figure 2.** DEAE-Sephadex performance chromatography (A); SDS-PAGE of xylanase purified by DEAE-sephadex (B) (Lane 1: crude supernatant; 2: gel filtration fraction; 3: molecular weight markers; 4-6 ion-exchange fractions)

**Table 1.** Summary of the purification of xylanase from *A. oryzae* VTCC F187.

Purification steps	Total activity (IU)	Protein (mg /mL) ( $\bar{x} \pm SD$ )	Specific activity ( $\bar{x} \pm SD$ )		Purification (fold)	Yield (%)
			IU/mL	IU/mg protein		
The culture supernatant	3611.3	1.57 $\pm$ 0.02	515.9 $\pm$ 4.0	328.6 $\pm$ 3.9	1.00	100.0
Sephadex G75	2313.5	0.47 $\pm$ 0.04	330.5 $\pm$ 5.4	703.2 $\pm$ 17.1	2.14	64.1
DEAE- sephadex	900.2	0.100 $\pm$ 0.001	128.6 $\pm$ 3.1	1286.1 $\pm$ 35.9	3.91	24.9

Based on the properties of the enzyme, each xylanase is purified by an appropriate purification method. In addition, the methods are often combined in a reasonable manner to improve purity as well as the yield. To purify xylanase from fungi, there are a variety of procedures, but generally, there is a combination of gel filtration chromatography and ion-exchange chromatography. In the world, most xylanase purification studies also use these two types of columns (Lu *et al.*, 2008), (Nair *et al.*, 2008) to obtain high purity xylanase but very low yield. Some studies use stronger negative ion-exchange chromatography columns, such as Mono (He *et al.*, 2015). In previous studies, our team also used Sephadex G100, G200 gel filtration chromatography and DEAE-sephadex ion-exchange chromatography to purify xylanase from some *Aspergillus* spp. such as *A. awamori* (Do *et al.*, 2012), *A. oryzae* DSM1863 and obtained enzymes with relatively high purification and yield (up to 11 times and 28% (Do, Quyen 2010).

In this study, we chose sephadex G75 as the material for gel filtration chromatography. Sephadex G75 has a protein molecular weight of 3–80 kDa which is more suitable for xylanase from *A. oryzae*, and also at a lower cost than other sephadex of the same type (G100, G200). Not only that, another advantage of sephadex is high recovery ability, which means can be reused after sterilization and preservation with disinfectant. The gel also has the lowest cost of all kinds of filter chromatography and is suitable for this type of xylanase when compared to superdex or sephacryl. This gives a quite significant cost advantage under experimental conditions in Vietnam. Next, we use the DEAE- sephadex, a negative ion-exchange chromatography, with a sample buffer with an ion force gradient (NaCl gradient) to increase the quality of protein separation.

As a result, we obtained xylanase with an increased purity of 3.91 times, a yield of 24.9% and a purified enzyme activity of 128.6 IU/mL. The results 3.91 times can be said to be a

modest result compared with similar studies in the country and around the world (Do, Quyen 2010), (He *et al.*, 2015), (Lu *et al.*, 2008), (Nair *et al.*, 2008). The reason may be that, firstly by the method, some foreign studies have combined the method of precipitation of ammonium sulfate salt (Lu *et al.*, 2008), (Nair *et al.*, 2008) to eliminate the trash protein; others use more sensitive types of chromatography such as the BioLogic DuoFlow pressure chromatography system (He *et al.*, 2015). Secondly, regarding the quality of the chromatographic system, studies mainly use pre-packed chromatographic columns such as MonoQ (He *et al.*, 2015), with a high-quality sample collection and analyzer system that can be used in large quantities. However, these methods and systems are very expensive, the cost can be higher a lot than our research. In addition, purified xylanase from these studies also has relatively low activity and yield of 28 IU/mL (Nair *et al.*, 2008), 21 IU/mL (Do *et al.*, 2012), 4-10% (He *et al.*, 2015). Our research results have purified xylanase with high yield and especially the activity has achieved significantly higher than similar studies and the cost of raw materials for purification is also lower. Therefore, overall, with an abundant source of xylanase such as *A. oryzae* VTCC F187, the initial results we obtained were relatively positive.

Regarding the molecular weight of xylanase, the results of the electrophoresis of the segments through our sephadex G75 column appeared two bands 55 kDa and 32 kDa. But after the second step, the electrophoresis of the segments with the highest xylanase activity was only darkened at about 32 kDa. Thus, it is possible to initially identify the purified xylanase in our study having weight mass of about 32 kDa. This result is also consistent with similar research results. The molecular weight of xylanase from *Aspergillus* sp. can range quite widely between 20–60 kDa. However, most of the xylanase purification results from *Aspergillus* sp. strains obtained enzymes with molecular weight of about 21–39 kDa (Do *et*

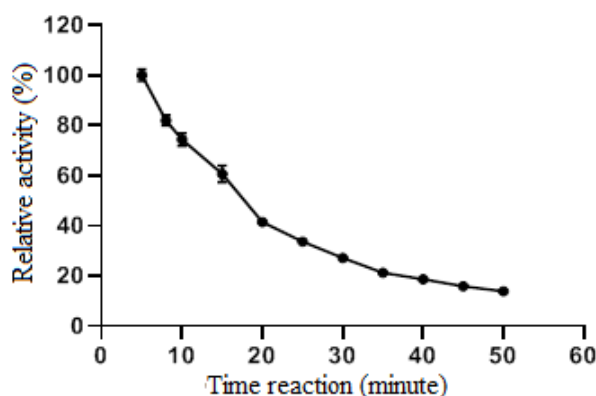
*al.*, 2012), (Chipeta *et al.*, 2005), (Do, Quyen 2010), (Golubev *et al.*, 1993), (He *et al.*, 2015), (Lu *et al.*, 2008), (Nair *et al.*, 2008).

**Effect of time on the activity of xylanase**

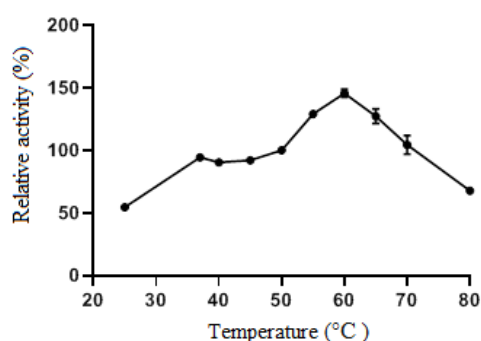
Xylanase activity reached a peak with the reaction time being 5 minutes then decreased almost linearly when increasing reaction time. Results showed that the optimal reaction time for xylanase from *A. oryzae* VTCC F187 was 5 minutes (Fig. 3).

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

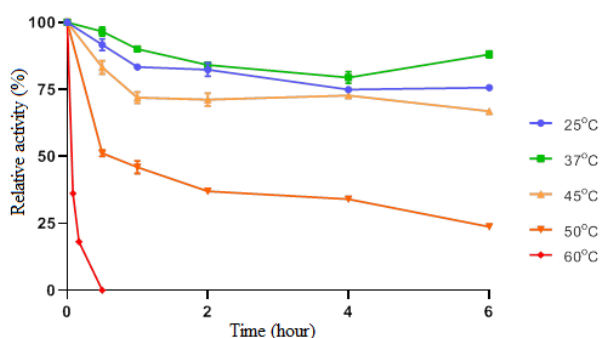
The optimum temperature of xylanase was found to be 60°C (Fig. 4). At 25°C and 80°C, xylanase retained 54.6 and 67.7% of normal activity. After 6 hours of incubation, the xylanase retained about 75% activity at 25°C and 37°C. For above 50°C, it was observed that the xylanase activity decreased significantly, especially, at 60°C, the xylanase lost all activity after incubated 0.5 hours (Fig. 5).



**Figure 3.** Effect of time reaction on the activity of xylanase from *A. oryzae* VTCC F187.



**Figure 4.** Effect of temperature on the activity of xylanase from *A. oryzae* VTCC F187.



**Figure 5.** Effect of temperature on the stability of xylanase from *A. oryzae* VTCC F187.

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

The optimum activity of xylanase was found to be at pH 6.5 in 50 mM potassium

phosphate buffer (Fig. 6). Xylanase retained more than 83% activity at pH range 3.0–5.0 after 6 hours incubated, at 25°C. After 6 hours, at 25°C, less than 75% activity was retained by xylanase at pH range 6.0–8.0. The xylanase

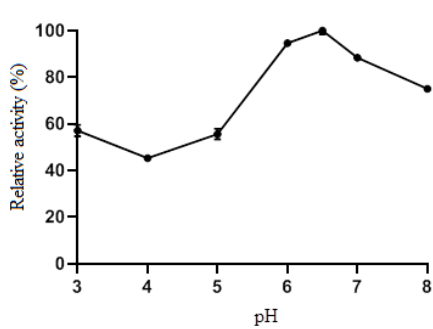
showed a broad pH activity profile, especially in pH acidity range (Fig. 7).

**Effect of inhibitors on the activity xylanase**

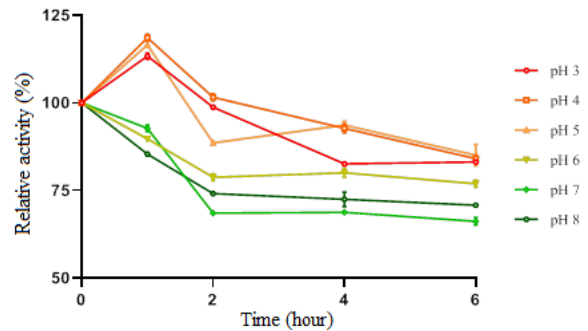
The agents EDTA at the concentration of 10 mM and SDS at the concentration of 2% showed a decrease in the activity of the xylanase from *A. oryzae* VTCC F187 by 56–77% in comparison to the original activity that means the enzyme required metal ions for its catalysis. Other four chemicals showed a slight decrease by about 15% after 1 hour of incubation (Fig. 8).

Firstly, the results show that xylanase from *A. oryzae* VTCC F187 has most of the characterizations similar to other xylanases in other studies. The first parameter, the optimum

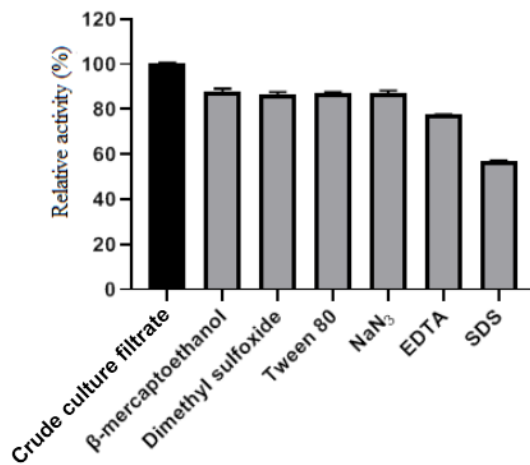
response time is 5 minutes (Do *et al.*, 2012), (Mellon *et al.*, 2010). The enzyme can be denatured by a high temperature reaction of 50–60°C when reaction time is longer. But if the reaction time is too fast (2–4 minutes), it can cause many errors due to enzyme substrate sample preparation and transferring to the incubator. The second parameter, the optimum reaction pH of 6.0–6.5 is within the optimal reaction pH range of most xylanases from *Aspergillus* sp. 5.0–6.5 (Do *et al.*, 2012), (Camacho, Aguilar 2003), Chipeta *et al.*, 2005), (Do, Quyen 2010), (He *et al.*, 2015), (Lu *et al.*, 2008). The thermal stability of xylanase in this study is about 25–37°C (activity remains above 75% after 6 hours of incubation), similar to some of our previous studies (Do, Quyen 2010), (Do *et al.*, 2012).



**Figure 6.** Effect of pH on the activity of xylanase from *A. oryzae* VTCC F187.



**Figure 7.** Effect of pH on the stability of xylanase from *A. oryzae* VTCC F187.



**Figure 8.** Effect of inhibitors on the activity xylanase from *A. oryzae* VTCC F187.

Secondly, purified xylanase from *A. oryzae* VTCC F187 in our study also has different properties compared to most xylanases from *Aspergillus* sp. in similar studies. The optimum reaction temperature of 60°C indicates that the xylanase is able to react better at higher temperatures than xylanases from *Aspergillus* sp. in many other studies (Krisana *et al.*, 2005), (Lu *et al.*, 2008), (Nair *et al.*, 2008) with the optimum reaction temperature between 40–55°C. This would likely be an advantage of the xylanase when competing in industries that require catalytic reactions at higher temperatures. Another property is its high stability in acidic pH (3–5). Generally, xylanase from *Aspergillus* sp. is stable in the range of pH 5.0–10.0 (Camacho, Aguilar 2003), (He *et al.*, 2015). Thus, pretty much the xylanase from fungi is stable in neutral or slightly alkaline medium, the xylanase from *A. oryzae* VTCC F187 is more stable in the acidic environment. This ability may be an important advantage when applied in acidic reactions at low pH. For example, in pulp bleaching. Finally, based on previous stability studies of xylanase, we selected several inhibitors to test the effect of xylanase. The results showed that xylanase from the studied strain was less affected by almost these substances (the remaining activity was up to 85% after 1 hour of incubation). There are two inhibitors 10 mM EDTA (nearly 75% active) and 2% SDS (v/v) (nearly 55% active) more powerful. From this result, it can be seen that this xylanase is more superior to some similar products: xylanase from *A. oryzae* DSM1863 was completely inhibited by SDS 2% (v/v), xylanase activity from *A. niger* DSM 1957 was only 36.2 % active when incubated in SDS and almost 50% incubated with dimethyl sulfoxide (Do *et al.*, 2009).

## CONCLUSION

In this study, we have successfully purified and characterized xylanase from *A. oryzae* VTCC F187. *Aspergillus oryzae* VTCC F187 xylanase showed superior heat and pH tolerance, therefore may have potential and

considerable applications in functional food and paper industries as well.

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## TINH SẠCH VÀ ĐÁNH GIÁ TÍNH CHẤT LÝ HÓA CỦA XYLANASE TỪ CHỦNG *ASPERGILLUS ORYZAE* VTCC F187

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<sup>3</sup>Trường Đại học Dược Hà Nội

### TÓM TẮT

Xylanase được sinh tổng hợp từ các loại vi khuẩn, nấm trong đó nấm *Aspergillus oryzae* đang là một đối tượng tiềm năng. Trong nghiên cứu này xylanase được tinh sạch từ dịch nổi của chủng nấm *Aspergillus oryzae* VTCC F187 sau 7 ngày lên men ở môi trường tối ưu bao gồm lõi ngô 7% và 5% bột đậu tương. Sau hai bước tinh sạch qua cột sắc ký lọc gel sephadex G75 và sắc ký trao đổi ion DEAE-sephadex, chúng tôi đã thu được xylanase tinh sạch có trọng lượng phân tử đạt 32 kDa được thể hiện trên điện di SDS-PAGE, với hiệu suất tinh sạch là 24,9%, độ sạch đạt 3,91 lần. Xylanase tinh sạch có hoạt tính đặc hiệu đạt 1268 IU/mg protein. Xylanase hoạt động tối ưu ở 60°C, pH 6,5 và enzyme vẫn hoạt động hiệu quả trong dải pH 3,0–5,0 và ở nhiệt độ dưới 37°C. Một số chất như  $\beta$ - mercaptoethanol, DMSO, Tween 80 ở nồng độ 2% và  $\text{NaN}_3$  ở nồng độ 10 mM làm giảm nhẹ, hoạt tính xylanase còn lại đạt trên 85%. Trong khi EDTA ở nồng độ 10 mM và 2% SDS ức chế mạnh sự hoạt động của enzyme xylanase, hoạt tính còn lại tương ứng đạt 77,6 and 56,6% so với đối chứng. Từ các số liệu về đánh giá tính chất lý hóa gợi ý xylanase là enzyme có ứng dụng tiềm năng,

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bao gồm việc sử dụng làm thức ăn chăn nuôi hoặc sử dụng thủy phân tạo ra các sản phẩm sinh học thân thiện với môi trường.

**Từ khóa:** *Aspergillus oryzae* VTCC F187, DEAE, sephadex, xylanase