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Purification and evaluation for effects of temperature on extracellular xylanase activity from *Aspergillus oryzae* DSM 1863

Tinh sạch và đánh giá ảnh hưởng của nhiệt độ lên hoạt tính enzyme xylanase ngoại bào của chủng Aspergillus oryzae DSM 1863

Research article

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Xylanase was purified from the crude culture of *Aspergillus oryzae* DSM1863 by sephadex G200 and DEAE – cellulose ion exchange chromatography. The molecular mass of the purified xylanase determined by SDS–PAGE was 21 kDa with a specific activity of 6768 U/mg towards 1% (w/v) of birch wood xylan. The optimum temperature was observed at 60°C. The enzyme was thermostable in the temperature range of 37-50°C with a high residual activity of 62-74% (650.6- 775.9 U/mg protein).

Enzyme xylanase được tinh sạch từ dịch lên men của chủng Aspergillus oryzae *DSM1863 sau khi qua cột sắc ký lọc gel sephadex G200 và sắc ký trao đổi ion DEAE – cellulose. Khối lượng phân tử của enzyme xylanase tinh sạch được xác định bằng điên di đồ SDS- PAGE. Xylanase tinh sạch có kích thước là 21 kDa với hoạt tính đặc hiệu đạt 6768 U/mg sau khi được xác định với nồng độ cơ chất là 1% birch wood xylan. Nhiệt độ tối ưu để enzyme hoạt động mạnh nhất là 60°C. Enzyme xylanase khá bền nhiệt. Hoạt tính của enzyme vẫn còn duy trì 62-74% (hoạt tính đặc hiệu đạt 650.6-775.9 U/mg protein) sau khi 8 giờ ủ ở 37-50°C.*

Keywords: *Aspergillus oryzae* DSM1863, purification, temperature, xylanase

1. Introduction

Xylan is a major ingredient of the hemicellulose complex. To hydrolyze xylan, the cooperative action of several enzymes of different functions is necessary. The enzyme involved in the degradation of main polysaccharide chain are endo-1,4-β-D-xylanase (EC 3.2.1.8) randomly cleaving the xylan backbone, β-D-xylosidase (EC3.2.1.37) cleaving xylose monomers from the non-reducing end of xylooligosaccharides. The removal of side groups is catalysed by α-L-arabinofuranosidases (EC 3.2.1.55), α-D-glucuronidases (EC 3.2.1.139), acetylxylan esterases (EC 3.1.1.72), ferulic acid esterases (EC 3.1.1.73) and p-coumaric acid esterases (EC 3.1.1.-) (Collins et al., 2005).

Endoxylanase plays a key role in xylan hydrolysis and have applications in various industries including pulp (Zhao et al., 2006; Ayyachamy and Vatsala, 2007), bread making (Jiang et al., 2005; Romanowska et al., 2006), brewery , beverage (Polizeli et al., 2005), xylitol, feed enzyme (Khandeparker and Numan, 2008). Xylanase is used for pulp industry as it supports the bleaching process, reduce the toxic chemicals (chlorine) used to bleach lignin contained in the paper. Other important applications of xylanase are to make the bread fine and soft and extend the storage time; to purify fruit juice, wine and beer; and to form xylitol glucose used in confectionary industry. In the breeding, xylanase expedites the digestion process of food containing xylan and at the same time helps to reduce the viscosity in the digestive system and follow by many positive effects such as improved food absorption, improved microorganism of the intestine in the advantageous direction, reduced digestion disorder and drier excrement (Bernier et al., 1983).

A variety of microbes, including bacteria, yeast and filamentous fungi, has been reported to produce xylanases, in which the most potent producers are fungi (Haltrich et al., 1996). A number of xylanases have been purified from a wide variety of microbes such as *Bacillus* strains (Bernier et al., 1983; Panbangred et al., 1985; Ayyachamy and

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Vatsala, 2007) and *Aspergillus* strains (Fernandez-Espinar et al., 1994; Fialho and Carmona, 2004; Krisana et al., 2005; Lu et al., 2008; Nair et al., 2008). However, xylanases are produced mainly by *Aspergillus* and *Trichoderma* sp. on an industrial scale. The fungus *Aspergillus oryzae* has been used in the tradition food industry of Japan for over 1000 years. As *A. oryzae* has been long used in food production, it is considered as a safe fungus and certified by World Health Organization (Machida, 2002). *A. oryzae* is able to secrete a large amount of enzyme in the environment. This property has been used in the commercial production for recombined enzyme and natural enzyme of *A. oryzae*. Many types of recombined protein are generated in submerged culture medium; however, in solid fermenting medium, *A. oryzae* as well as other types of fungus, is able to generate a large amount of enzyme which is higher than that generated in the submerged culture medium. In Japan, many types of commercial enzyme have been produced by solid fermentation as well as by conventional fermentation (Tsuchiya et al., 1994).

Here, we purified and characterization of xylanase from *A. oryzae* DSM1863. We also identified the optimum temperature and thermostable of the purified xylanase. The biochemical characteristics suggested that the xylanase has a potential application, including use as a feed enzyme.

2. Materials and methods

2.1 Strain and culture conditions

The *Aspergillus oryzae* DSM1863 purchased from the German Microorganism Collection Center (DSMZ) was grown on the liquid medium containing 2 g/l $NaNO_3$; 1 g/L K_2HPO_4; 0.5 g/L $MgSO_4$; 0.5 g/L KCl; 10 g/L soybean powder; 40 g/L corn cobs; pH 7.0. The inoculated flasks were incubated for 4 days at 30°C on a rotary shaker at 200 rpm.

2.2 Chemicals

Birch wood xylan was purchased from Biochemika; 3.5-dinitrosalicylic acid (DNS) from Fluka. Sephadex G200 and DEAE- cellulose were supplied by Pharmacia Co. (GE Healthcare. SDS was supplied Sigma Aldrich Co. (St, Louis, USA). Agar, soybean powder and corn cobs were from Biotech (Vietnam). $NaHPO_4$; Na_2HPO_4; $NaNO_3$ and $MgSO_4$ were from BioBasic Inc (Ontario, Canada). All other chemicals were of analytical grade unless otherwise stated.

2.3 Method

2.3.1. Xylanase purification

The culture was centrifuged for 10 min, at 8000 xg. Four ml of the crude enzyme extract (114.3 units) was applied to a Sephadex G-200 column (2.6 x 6 cm) pre-equilibrated with 50 mM potassium phosphate buffer pH 7.5 at a flow rate of 25 ml/h then washed with the same buffer. The eluate was collected with 18 ml per fraction. A highly active xylanase pool of 5 ml through Sephadex G-200 column was further applied to a DEAE-cellulose ion exchange chromatography pre-equilibrated with 50 mM Tris HCl buffer pH 8.0 containing 50 mM NaCl (buffer A), then washed with the same buffer. The protein was eluted with 50 mM Tris HCl buffer pH 8.0 containing 1000 mM NaCl (buffer B) at a flow rate of 20 ml/h until $OD_{280nm} < 0.010$. The eluate was collected with 7.5 ml per fraction. The fractions containing highly active xylanase activity were pooled and used as purified enzyme for characterization. All purification steps were carried out at 4°C, unless otherwise specified.

2.3.2 Xylanase activity estimation

Xylanase activity was determined by measuring an increase of concentration of reducing sugars produced by enzymatic hydrolysis of birchwood xylan. A reaction mixture of 100 µl of the crude or purified xylanase containing 0.1 µg total protein was incubated with 400 µl of 0.5% (w/v) birch wood xylan in 20 mM potassium phosphate buffer pH 6.5 at 55°C for 5 min. To arrest the reducing sugar released in the reaction mixture, 1.25 ml of 3.5,-dinitrosalicylic acid (DNS) was added. The reduced sugars were determined by measuring the absorbance at 540 nm (Miller, 1959). D-xylose was used as standard. One unit (IU) of xylanase activity was defined as the amount of enzyme that released 1 µmol of xylose per min under the standard assay conditions. All measurements were carried out three times and from these values the average value was taken.

2.3.3 SDS-PAGE and protein concentration

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli (Laemmli, 1970) with Bio-Rad equipment. SDS-PAGE was usually performed on gels containing 12.5% (w/v) acrylamide according to the manufacture's recommendation. The gels were stained with Coomassie Brilliant Blue R- 250 for protein. Protein was estimated by the method of Braford with the bovine serum albumin as standard (Bradford, 1976).

2.3.4 Effects of temperature on xylanase activity and thermostability

To investigate the effect of the reaction temperature on the xylanase activity, 100 µl of the purified enzyme (0.1 µg protein) was incubated together with 400 µl of 0.5% (ww/v) of birch wood xylan in 20 mM potassium phosphate buffer pH 6.5 at different temperatures 37°C; 40°C; 45°C; 50°C; 55°C; 60°C, and 70°C for 5 min and then the reducing sugars were arrested by addition of 1.25 ml DNS reagent. To study the thermostability of the enzyme, 10µl of the purified xylanase (0.1 µg protein) were incubated at temperatures ranging from 37°C; 50°C; 60°C to 70°C for different intervals of time before using it for assay.

3. Results

3.1. Purification of xylanase

The xylanase production by *A. oryzae* DSM1863 in the mineral medium was 114.3 U/ml (specific activity of 615.04 U/mg protein) after 72 hours of cultivation. This culture supernatant was applied to Sephadex G-200 and DEAE- cellulose chromatography. The pooled Sephadex G-200 fractions containing high xylanase activity (3768

IU/mg) were applied further to the DEAE- cellulose ion exchange chromatography (Fig. 2). The xylanase was purified with a factor of 11 and a yield of 28%. The purified enzyme gained a specific activity of 6768 IU/mg (Tab. 1) and showed a unique protein band on SDS-PAGE (Fig. 2). The molecular weight of this protein was estimated to be 21.0 kDa.

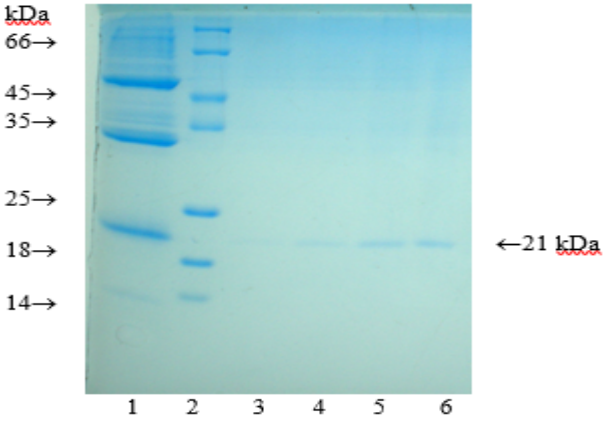

Figure 1. SDS-PAGE of the purified xylanase from *A. oryzae* DSM1863 through Sephadex G-200 and DEAE-cellulose (lane 1: the crude enzyme; lane 2: molecular weight marker; lane 3-6: the fractions were purified.

Nair et al (2008) purified two xylanases I and II from crude culture filtrate of *A. sydowii* SBS45 through a three-step purification scheme involving ammonium sulfate precipitation, gel filtration chromatography (Sephadex G-200), and anion exchange chromatography (DEAE-Sephadex A-50). Xylanase I and II were purified 93.41 and 77.40 times with yields of 4.49 and 10.46, respectively and had molecular weights of 20.1 and 43 kDa, respectively on SDS-PAGE.

Lu et al. (2008) purified a xylanase from *A. ficuum* AF-98 throughout the precipitation with 50-80% $(NH_4)_2SO_4$, DEAE-Sephadex A-50 ion exchange chromatography and Sephadex G-100 chromatography. The extracellular xylanase from this fungal was purified 32.6-fold to homogeneity and had a specific activity of 289 U/ mg protein and showed a monomeric protein with a molecular mass of 35 kDa as determined by SDS-PAGE (Lu et al., 2008).

Fialho and Carmona (2004) also obtained two proteins with molecular weight of 21 kDa and 24 kDa during the purification of xylanase from *A. giganteus* using ammonium sulfate and Sephadex G-75. The purified xylanase from *A. nidulans* showed a single band on SDS-PAGE with a molecular mass of 34 kDa and had an isoelectric point of approximately 3.4 (Fernandez-Espinar et al., 1994). The xylanase from *Aspergillus* cf. *niger* was purified using DEAE Sepharose and Phenyl Sepharose 6 Fast Flows to a homogeneity and showed a molecular mass of 21 kDa (Krisana et al., 2005). An extracellular 22-kDa xylanase produced by *Aspergillus* sp. FP-470 strain was purified 30-fold (Camacho and Aguilar, 2003).

Table 1. Purification of xylanase from *Aspergillus oryzae* DSM1863

Steps	Total protein (mg/ml)	Total activity U/ml	Specific activity (U/mg)	Purification factor	Yield (%)
Crude enzyme	0.1858	114.3	615.04	1	100
Sephadex G200	0.0212	78.7	3768	6.13	43
DEAE-Sephadex	0.0126	84.3	6768	11	28

3.2. Optimum temperature and thermostability

The xylanase from *A. oryzae* DSM1863 acted in a large temperature range from 40°C to 70°C. The xylanase activity increased gradually from 82% (744.6 U/mg) at 40°C to the maximum of 100% (903.1 U/mg) at 60°C (Fig. 2) and then decreased gradually to 82% (738.3 U/mg) at 70°C. The temperature treatment from 37°C to 70°C for 1-8 hours showed an obvious temperature effect on the xylanase stability. The xylanase from *A. oryzae* DSM1863 possessed a thermostability in a temperature range 37-50°C.

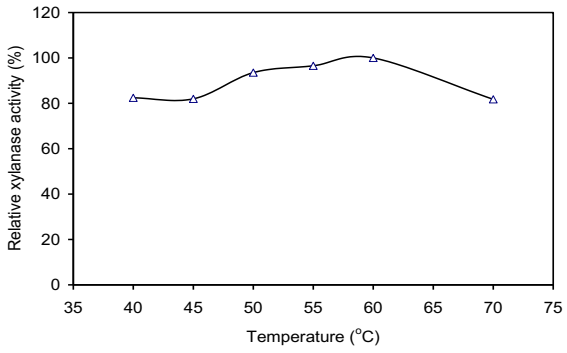

Figure 2. Effect of temperature on xylanase activity

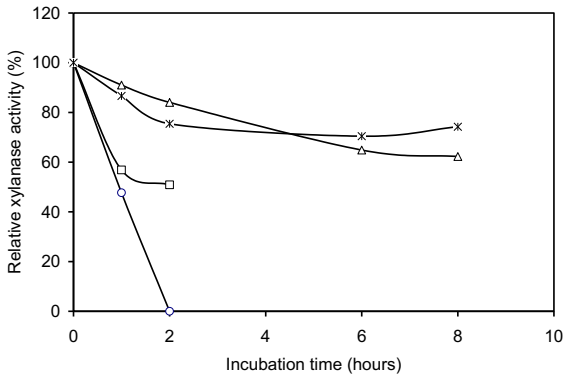

Figure 3. Effect of stability temperature on xylanase activity (rhombus: 70°C; squares: 60°C; triangles: 50°C; stars: 37°C)

The residual activity profiles of the xylanase treated at 37°C and 50°C were quite similar (Fig. 3). The residual xylanase activity decreased gradually to 62% (650.6 U/mg) and 74% (775.9 U/mg) for 8 hours of the temperature treatment at 50°C and 37°C, respectively, whereas the xylanase activity lost one half when it was treated just for 1 or 2 hours at 70°C or 60°C, respectively.

The xylanase from various *Aspergillus* strains had different optimum temperatures however the optimum temperatures ranged from 45°C to 60°C. The optimum temperature was 60°C (XynF1 from *A. oryzae* (Kitamoto et al., 1999)), 55°C (endo-1,4-β-xylanase B from *Aspergillus* cf. *niger* BCC14405)(Krisana et al., 2005), 50°C (xylanase from *A. giganteus*) (Fialho and Carmona, 2004), the xylanase I and II from *A. sydowii* SBS 45 (Nair et al., 2008), 45°C (xylanase from *A. ficuum* AF-98 (Lu et al., 2008).

4. Conclusions

A xylanase was purified from the culture supernatant of *A. oryzae* DSM1863 throughout Sephadex G-200 chromatography and DEAE-cellulose ion exchange chromatography. The purified xylanase showed a specific activity of 6768 IU/mg protein and a protein band of about 21 kDa on SDS-PAGE. The enzyme showed the optimum temperature was observed at 60°C. The purified xylanase was stable in the temperature range 37-50°C.

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