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CHARACTERIZATION OF *STREPTOMYCES SP.* 45I-3 XYLANASE

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ABSTRACT

Streptomyces sp 45I-3 producing xylanase was isolated from Kalimantan soil sample. Crude enzyme (produced by centrifuging the culture at 14000 rpm for about 5 minutes) and purified xylanase has an optimum condition at pH 5 and 50 °C. Crude xylanase have half-life time of 2 hours 9 minute at 50 °C, whereas purified xylanase has half-life time of 12 hours. The molecular mass of purified xylanase was determined to be 43.2 and 39.2 kDa.

Key words: Xylanase, Characterization, Streptomyces.

INTRODUCTION

Xylan is a complex heteropolysaccharide comprising a backbone of xylose residues linked by â-1,4 D-xylose glycosidic bond (Saha 2002, Tseng *et al.* 2002). This backbone can be substituted with side-chain of O-acetyl, á-L-arabynofuranosyl, D-glucuronyl, and O-methyl-D-glucuronyl residues (Kubata *et al.* 1994, Silveira *et al.* 1999, Saha 2003).

A complex enzyme is needed to hydrolyse xylan to its monomer. This complex enzyme includes endo-b-1,4 xylanase dan b-xylosidase for hydrolisis of the back bone, while the side-chain is liberated by a-arabynofuranosidase, a-D-glucuronidase, a-galactosidase and acetyl xylan esterase. The enzymes work synergistically in hydrolysing xylan (Sunna *et al.* 1997, Subramaniyan & Prema 2002, Ali *et al.* 2004).

Numerous applications in the industries rapidly grow the use of xylan and xylanase. One of the most popular usage of xylanase is in pulp industry to reduce the amount of chlorine for bleaching. Xylanase used in pulp industry should have an activity

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under alkaline condition (Wong *et al.* 2004). Xylanase is also used for clarifying juices, wine and extracting coffee, plant oils, and starches. Recently, xylanase is also used to produce monomer sugar (xylose) and diet-fiber such as xylotriose, xylotetraose (Beg *et al.* 2001, Adeola & Bedford 2004).

In our previous research we had identified xylanolytic *Streptomyces sp* 45I-3. This research will characterize and purify this enzyme.

MATERIALS AND METHODS

Stock culture and Inoculum Preparation

Streptomyces sp. 45I-3 isolate was rejuvenated in YM agar media (0.4% yeast extract, 1% Malt extract, 1.5% glucose, 1.5% agar). Inoculum was prepared by growing *Streptomyces* sp. 45I-3 on xylan medium (1% yeast extract, 10.3% sucrose, 0.5% birchwood xylan, 1.5% agar). Incubation was done at 30 ÚC for 7 days.

Xylanase Production

As much as 2 cockbors of isolate grown in xylan medium (the same medium as preparation of inoculum) was inoculated to 100 ml production media in 500 ml flasks. They were incubated with 140 rpm agitation at 30 ÚC for 10 days. Crude extract of xylanase was harvested every day by centrifuging the culture at 14 000 rpm for about 5 minutes. Xylanase activity was determined by measuring the xylose production from birchwood xylan as the substrate (do Nascimento *et al.* 2003). Xylose was detected by using DNS (Dinitrosalisilic Acid) method (Miller 1959). The yielded reducing-sugar was assessed by spectrophotometer at a wavelength of 540 nm. One unit of xylanase activity was defined as the amount of enzyme that released 1 μmol of xylose per ml of sample per minute in assay condition. Protein concentration (mg/ml) was measured by using Bradford method (1976). Bovine serum albumin (BSA) was used as a standard.

Purification

Enzyme purification was started by precipitation using an anionic polymer Eudragit S100 (metyl methacrylic : methacrylic acid = 2 : 1) according to Breccia *et al.* (1998) with modification. The enzyme was eluted by incubating the suspension with 1 M NaCl and 0.2 % (v/v) Triton X-100 in 0.2 M Tris HCl buffer pH 7.0 for 20 minute at 25 °C. The suspension was added with acetic acid 2M until the pH was 4 and incubated for 10 minutes at 25 °C and centrifugated at 4500 rpm for 15 minutes at 4 °C. The enzyme was concentrated with polyethylene glycol (PEG 6000) and dialyzed overnight with 0.2 M Tris HCl buffer pH 7.0. The dialyzed enzyme was loaded on Sephadex G100 and washed with the same buffer. Native PAGE was done according to Ornstein (1964) and Davis (1964) and the SDS PAGE according to Laemmli (1970).

Xylanase characterization

Characterization of crude extract and the purified enzyme included the determination of optimum temperature and pH, enzyme stability and molecular weight, as well as substrate specificity and kinetic parameters. The assessment with various pH was carried out within pH 3.0-9.0 at intervals of 0.5. The determination of optimum temperature was done from 30 until 90 ÚC at intervals of 10 ÚC. The stability of xylanase was tested by incubating the enzyme without substrate at the optimum temperatures.

Substrate specificity

The substrate specificity of xylanase was assayed using *p*-nitrophenyl-b-D-xylanopyranoside, *p*- nitrophenyl-a-L-arabynofuranoside, 4- nitrophenyl-acetate, 4- nitrophenyl -a-D-galactopyranoside and *p*- nitrophenyl -b-D-glucoropyranoside according to Saha (2001).

Kinetic parameters

Kinetics parameters were determined by incubating the enzyme with different amount of substrate. Xylanase was incubated with birchwood xylan (0.10-0.25 %) in citrate phosphate buffer of pH 5 at 50 °C. The values of the Michaelis constant were determined from Lineweaver-Burk plots.

RESULTS AND DISCUSSION

Production of xylanase

The daily production curve of xylanase *Streptomyces sp.* 451-3 tested at pH 7.2 and temperature of 37 °C is shown in Figure 1. The highest xylanase production was reached on day-8 with the activity of 8.3 U/ml. This optimum time of xylanase production was then used as the standard harvest time for the next xylanase production.

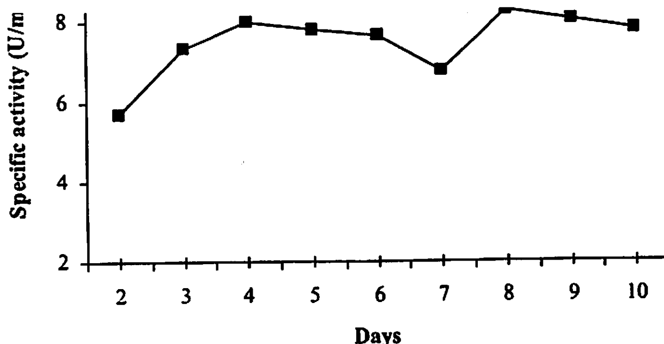

Figure 1. Production curve of *Streptomyces sp.* 451-3 xylanase measured at 37 oC and pH 7.2.

Effect of pH on xylanase activity

Figure 2 shows the effects of pH on the crude xylanase activity measured at 37 °C and purified xylanase activity measured at 50 °C.

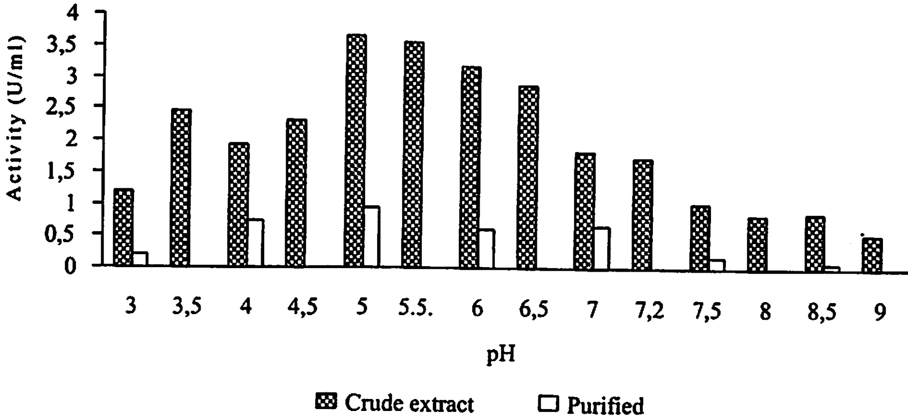

Figure 2. Effect of pH on *Streptomyces sp* 451-3 crude xylanase activity measured at 37 °C and purified xylanase activity measured at 50 °C.

Crude and purified xylanase had its highest activity at pH 5, but also demonstrated quite high activity at pH 5.5 to 6.5. Enzyme has specific optimum pH, which is the pH causing maximum enzyme activity (Lehninger 1982). The characteristic of enzyme's optimum pH is the condition where the catalytic site of the enzyme is at the expected ionization level (Whitaker 1994).

Effect of temperature on xylanase activity

Figure 3 shows the effects of temperature on the activity of xylanase tested at pH 5. Xylanase displayed its optimum temperature at 50 °C. Temperature fluctuation can influence the integrity of secondary, tertiary and quartenary structure of enzyme that can affect the enzyme activity (Whitaker 1994).

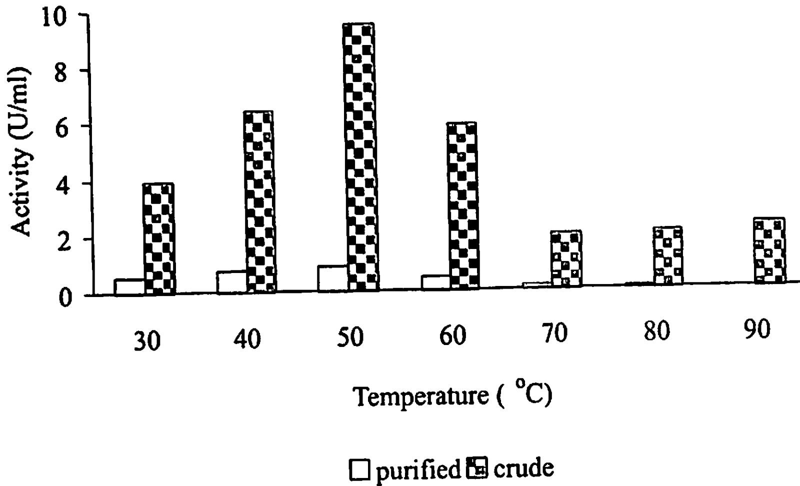

Figure 3. Effect of temperature on *Streptomyces sp* 45I-3 crude extract and purified xylanase activity measured at pH 5.

Enzyme Stability

At the optimum temperature, the crude enzyme have half-life time about 2 hours and 9 minutes whereas the purified enzyme about 12 hours (Figure 4). Enzyme stability was affected by protein, carbohydrate and cations on the medium. Purified enzyme was more stable than the crude extract which may be due to the lost of cations and other protein that function as an inhibitor for the enzyme. Enzyme thermostability is also due to the enzyme ability to maintain its three dimensional structure (Whitaker 1994).

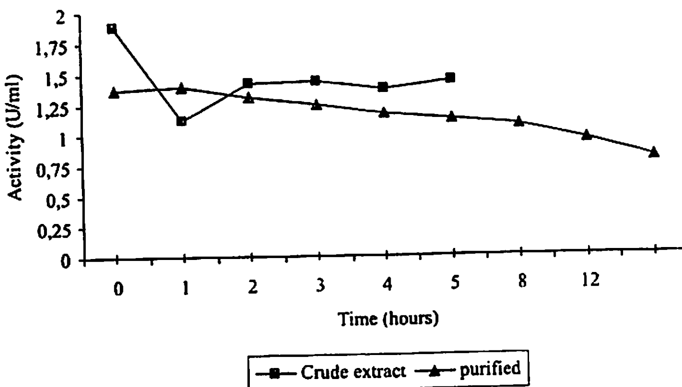

Figure 4. Stability of crude and purified xylanase on 50 °C

Purification

Eudragit S100 was able to bind 67.98% of the xylanase crude extract (Table 1). The enzyme was eluted by one molar NaCl and 0.2 % Triton X100 in 0.2 M Tris HCl pH 7.0 buffer 62.98 %. Purification using Eudragit S100 increased the purity about 12 times. Gupta *et al.* (1994) reported that using Eudragit S100 could just increase the purification factor of 4.2 for *Trichoderma viridae* xylanase, whereas Sardar *et al.* (2000) reported, the purification factor of 65 was observed for purification of *Aspergillus niger* xylanase. The xylanase binding ability to anionic polymer Eudragit S100 depends on the isoelectric point (pI) of the xylanase and the character of anionic polymer Eudragit S100.

Table 1. Purification of *Streptomyces sp.* 451-3 xylanase

Steps	Total Volume (ml)	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Recovery Yield (%)	Purification factor
Crude extract	30	33.46	1.0582	31.62	100	-
Eudragit S100						
- Supernatan	40	10.38	0.84	12.38	31.02	
Elution	22	21.08	0.06	380.45	62.98	12
Sephadex G100	8	7.52	0.01	817.18	22.47	26

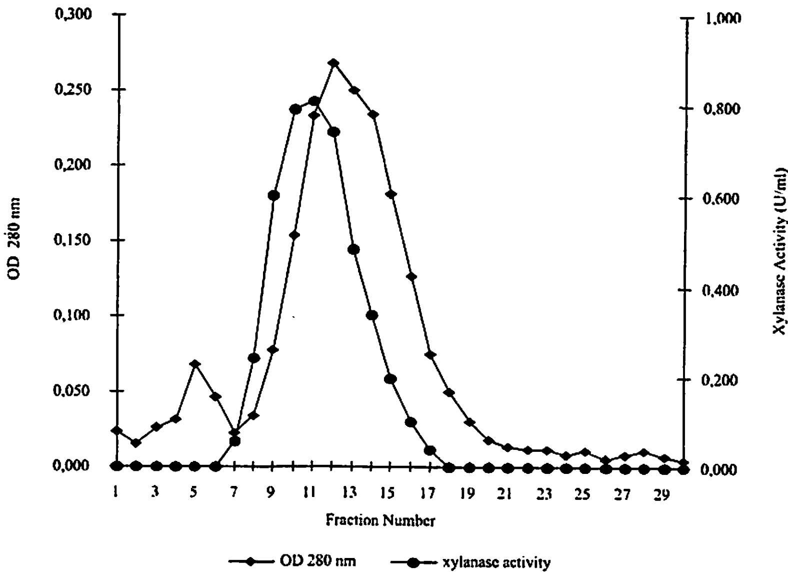

Figure 6. Profile xylanases chromatography elution with Sephadex G100 matrix

The result of purification using Sephadex G100 showed 2 peaks of protein, but the xylanase activity only displayed on the second peak (Figure 6). After using gel filtration with Sephadex G100, the purification factor increased to 26 times. SDS PAGE analysis showed two protein bands with the molecular mass of 39.2 and 43.2 kDa, respectively. The native PAGE also had two protein bands (Figure 7).

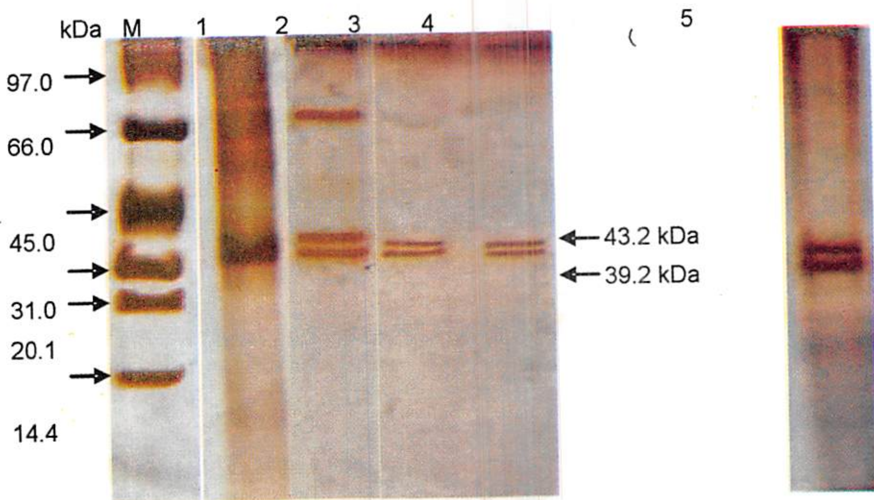

Figure 7. Profile of SDS PAGE of the purified xylanase from (1) crude extract (2) Eudragit S100 fraction (3-4) Sephadex G100 fraction 11-12, (M) *Low Molecular Weight* standard (Sigma) and (5) profile of native PAGE of the purified xylanases from Sephadex G100 fraction 11-12

Substrate specificity

Assays with substrate specific showed that this purified enzyme had an activity on *p*-nitrophenyl-b-D-xylanopyranoside or have a b-xylosidase activity (Table 3). *Streptomyces* have produced different xylanase. Kaneko *et al.* (2000) reported that *S. olivaceoviridis* E 86 has 23 kDa endoxylanase, whereas Belfaquih *et al.* (2002) reported that *Streptomyces achromogenes* 5028, *Streptomyces logisporus ruber* 4-167 and *Streptomyces sp.* 8812 have 25, 45 and 22 kDa endo-xylanase. *Streptomyces actuosus* A-151 has 4 â-xylanase with molecular mass of 30, 45, 26 dan 20 kDa, respectively (Wang *et al.* 2003).

Table 3. Substrate specificity of xylanase *Streptomyces sp* p. 45I-3 at pH 5 and 50°C

Specific Substrate	Activity (U/ml)
p-nitrophenyl-β-D-xylanopyranoside	0.11
p-nitrophenyl-α-D-arabynofuranoside	ND
p-nitrophenyl-β-D-glucuropyranoside	ND
p-nitrophenyl-α-D-galactopyranoside	ND
4-nitrophenyl-acetate	ND

Notes:
ND : not detected

Kinetics parameters

Reciprocal plots showed apparent Km and Vmax values of 9058 U/mg and 7.43 mg/ml.

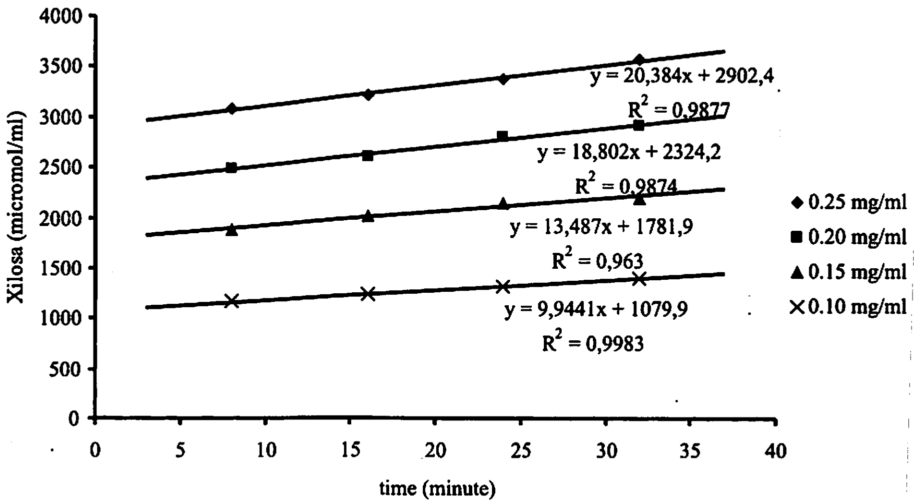

Figure 8. Xylose content on several Birchwood xylan concentration after incubation at pH 5 and 50 °C

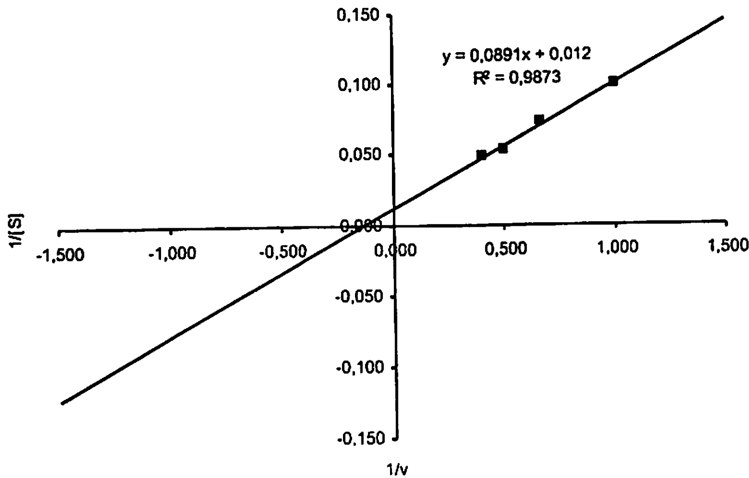

Figure 9. Correlation between 1/[S] and 1/[V]

Streptomyces achromogenes 5028, *Streptomyces logisporus ruber* 4-167 and *Streptomyces sp.* 8812 endo-xylanase have V_{maks} of 2198 ± 46, 2269 ± 32 and 302 ± 17.5 IU/mg with K_M = 5.0, 6.1 and 3.3 mg/ml, respectively (Belfaquih *et al.* 2002). *Streptomyces sp.* S38 that produced one endo-xylanase and two arabinofuranosidase have a V_{maks} value of 5700 ± 600, 620 ± 30 dan 1050 ± 50 IU/mg with K_M = 2.22, 1.05 and 0.97 mg/ml, respectively (Georis *et al.* 2000).

CONCLUSIONS

It is possible to purify xylanase from *Streptomyces sp* 45I-3 using Eudragit S100 and gel filtration. Crude and purified enzyme have the same optimum condition: pH 5 and 50 °C but the purified enzymes were more stable at the optimum condition: pH 5 and 50 °C.

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