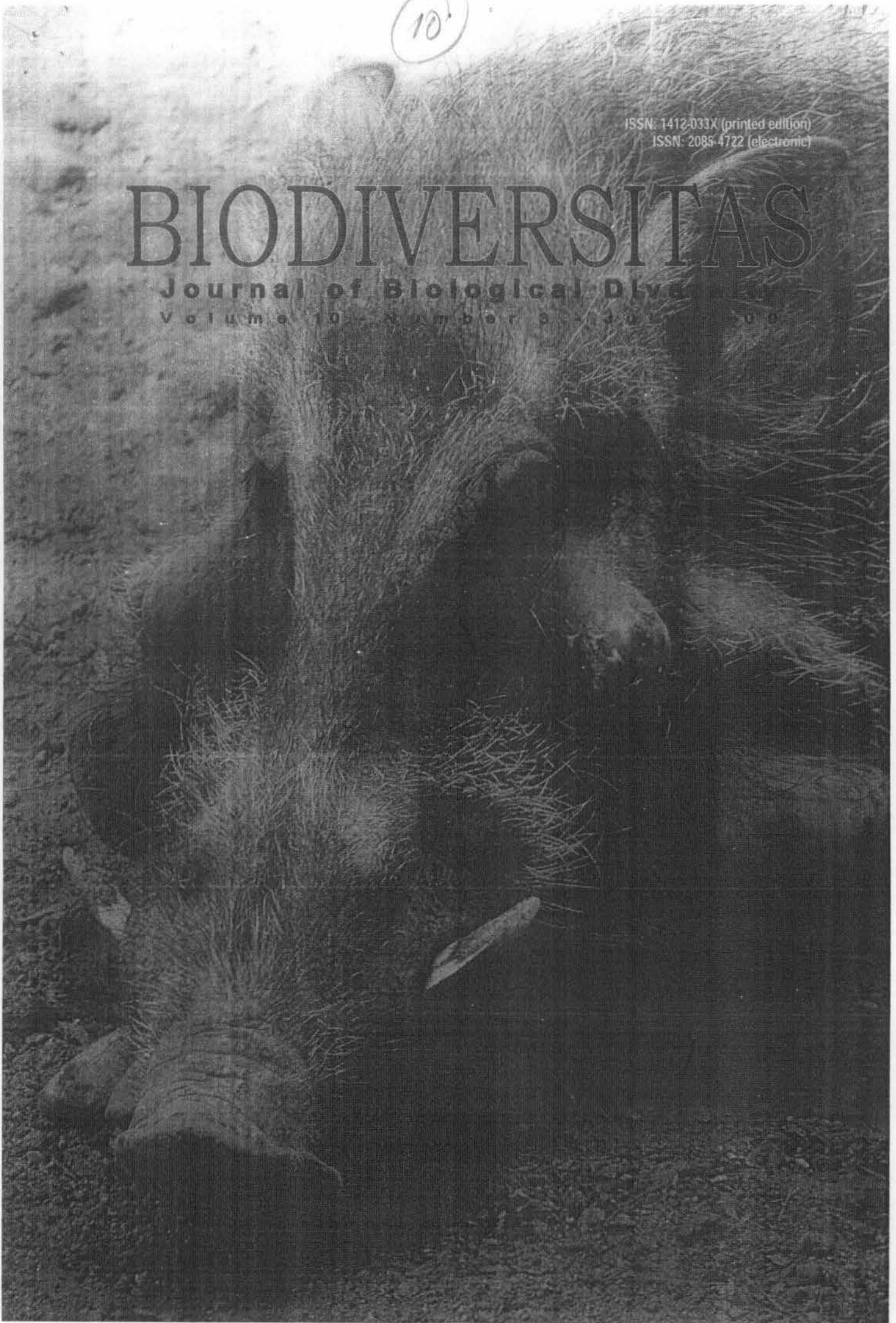


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Sus verrucosus
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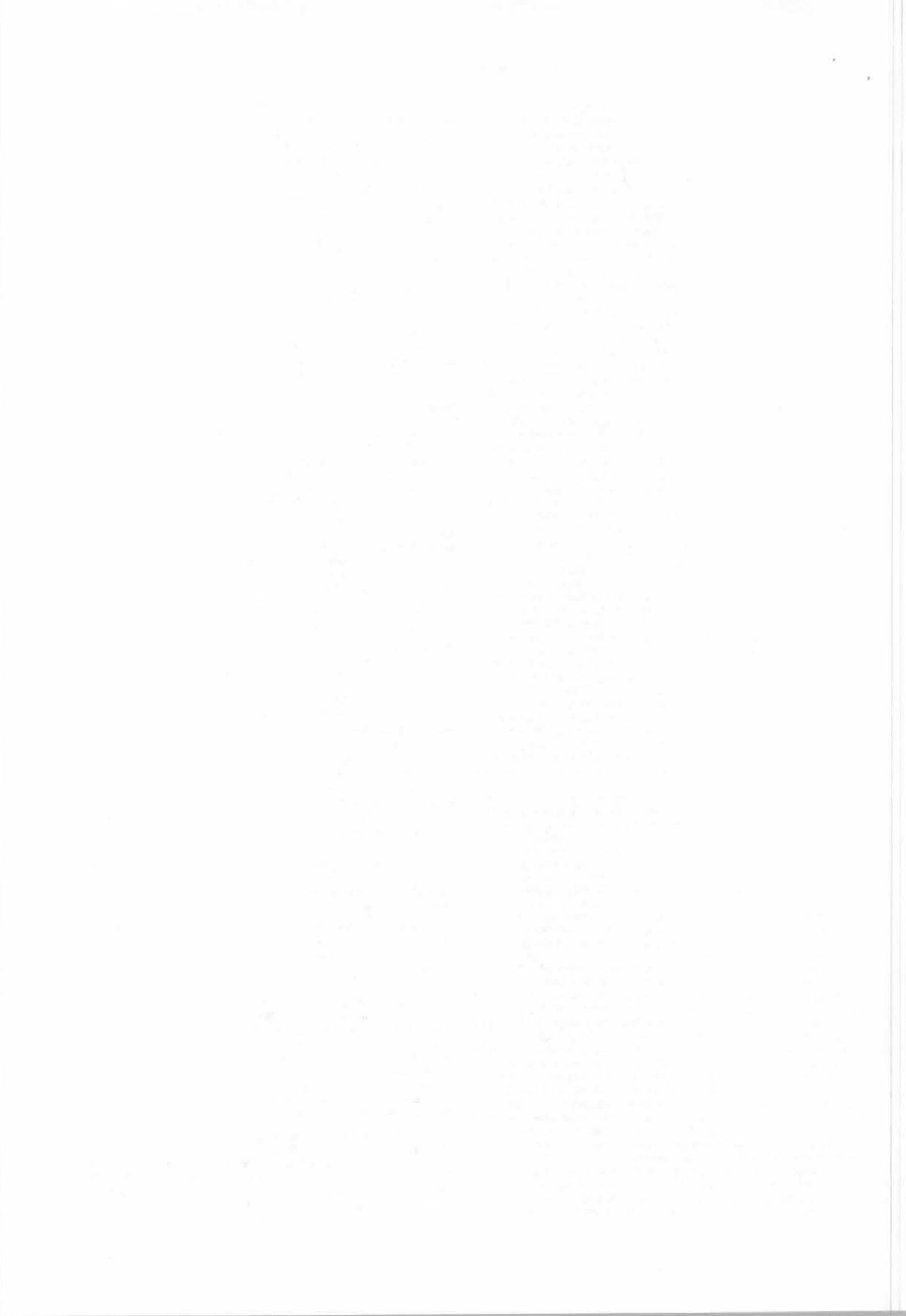
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Characterization and Purification a Specific Xylanase Showing Arabinofuranosidase Activity from *Streptomyces* spp. 234P-16

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ABSTRACT

Streptomyces spp 234P-16 producing xylanase was isolated from soil sample from Padang, West Sumatra, Indonesia. Crude enzyme (produced by centrifuging the culture at 14000 rpm for about 5 minutes) and purified xylanase have an optimum condition at pH 5 and 90°C. Crude xylanase have half life time of 4 hours, whereas purified xylanase have half life time of 2 ½ hours at 90°C. The molecular mass of purified xylanase was determined to be 42.4 kDa. The Arabinofuranosidase have a Km and Vmax value of 1,98 mg/mL and 523 µmol/minute/mg, respectively

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Key words: xylanase, xylooligosaccharide, arabinofuranosidase, *Streptomyces*.

INTRODUCTION

The utilization of hemicellulosic sugars is essential for efficient conversion of lignocellulosic materials to ethanol and other value-added products. Xylan, a component of hemicellulose, is a complex heteropolysaccharide comprising a backbone of xylose residues linked by β-1,4 D-xylose glycosidic bond (Saha 2000; Saha 2002; Tseng et al., 2002) with side-chain of O-acetyl, α-L-arabinofuranosyl, D-glucuronyl, and O-methyl-D-glucuronyl residues (Kubata et al., 1994; Silveira et al., 1999; Saha 2003). Xylan degrading enzymes includes endo-β-1,4 xylanase dan β-xilosidase and the side-chain are liberated by α-arabinofuranosidase, α-D-glucuronidase, galactosidase and asetil xylan esterase. These enzyme system work synergistically in hydrolysing complex xylan (Sunna et al., 1997; Subramaniyan and Prema, 2002; Ali et al., 2004). Commercial hemicellulase preparation need to be enriched with several accessory enzymes including arabinofuranosidase to be more effectively convert

hemicellulose to simple sugars.

Arabinofuranosidases hydrolyze terminal nonreducing residues from arabinose-containing polisaccharides. Arabinofuranosidases warrant substantial research effort because they represent potential rate limiting enzymes in xylan degradation, particularly those substrate from agricultural residues such as corn fiber, corn stover and rice straw. With the increase of enzyme usage in feed formulations, arabinofuranosidase will be important in the enhancement of feed digestibility.

In our previous research we identified xylanolytic *Streptomyces* sp 234P-16 that was isolated from soil sample in Padang West-Sumatra. In the present paper we report characterization and purification of enzyme xylanase.

MATERIALS AND METHODS

Stock culture and inoculum preparation

Streptomyces 234P-16 isolate was rejuvenated in YM agar-agar media (0.4% yeast extract, 1% malt extract, 1.5% glucose, 1.5% agar-agar). The isolate was then grown in xylan medium (1% yeast extract, 10.3% sucrose, 0.5% Birchwood xylan, 1.5% agar-agar). Incubation was done at 30 °C for 7 days.

Determining the optimum time for xylanase production and activity

As much as 2 cockbores of isolate grown in xylan medium was inoculated to 100 mL production

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medium (the same medium as preparation of inoculum) in 500 mL Erlenmeyer. They were incubated with 140 rpm agitation at 30 °C for 10 days. Crude extract of xylanase was yielded every day by centrifuging the culture at 14000 rpm for about 5 minutes. Activity of the crude enzyme was measured by using DNS (Dinitrosalicylic Acid) method by Miller (1959) with xylose used as standard. The yielded reducing-sugar was assessed by spectrophotometer with a wavelength of 540 nm. One unit xylanase activity was defined as the amount of enzyme which produces 1 μ mol xylose during 1 minute. Protein concentration (mg/mL) was defined by using Bradford method (Bradford 1976). The standard protein used was *bovine serum albumin* (BSA).

Xylanase characterization

Characterization of crude enzyme and the purified enzyme included the determination of optimum temperature and pH, enzyme stability. The assessment with various pH was carried out within pH 3.0-9.0 with intervals of 0.5. The determination of optimum temperature was done from 30 until 90°C with the intervals of 10°C. The stability of xylanase was tested by incubating the enzyme without substrate in the optimum temperatures.

Hydrolysis analysis using HPLC

The enzyme was suspended in 5 mL Birchwood Xylan 0.5% (w/v) and incubated at optimum condition (50°C, pH 5) for 5 hours, centrifuged at 10.000 rpm, 4°C and the supernatant was analyzed in HPLC (Waters, USA) with the condition: column: carbohydrate, solvent: 60% (v/v) methanol, detector: refraction index.

Precipitation of xylanase crude extract with acetone

The xylanase crude extract was precipitated by using several percentage of acetone concentration to know the optimum condition of xylanase fractionation by acetone. Table of acetone precipitation used was based on Scopes (1987). The percentage of the precipitation used varied from 60-90%.

Purification

The precipitated crude enzyme was purified using DEAE-Sephadex A-50 matrix (Sigma, USA) and Sephadex G-100 matrix. For the DEAE-Sephadex A-50 matrix, the washing buffer were 0.02 M buffer Tris-HCl pH 8.0. and the elution buffer was 0.0-0.5 NaCl in 0.02 M Tris-HCl buffer pH 8.0. The fraction containing xylanase activity was pooled and concentrated using PEG6000 and dialysis for 4 hours with 0.2 M phosphate buffer pH 7 before applied to the gel filtration. For the gel filtration 0.02 M phosphate buffer with pH 7.0 was used. The flow rate for chromatography was 0.5 mL/minute

Analysis of SDS-PAGE and Zymogram

Protein electrophoresis was conducted by using Laemmli methods (1970) with acrylamide concentration of 4% for collector gel and 10% for separator gel and 0.5% substrate was added for zymogram. As much as 20 μ L of crude extract of the enzyme from acetone 70% precipitation and the purification fraction from gel filtration and anion exchange was inserted to the well of SDS-PAGE.

Substrate specificity

The substrate specificity of xylanase was assayed using *p*-nitrophenyl- β -D-xylanopiranoside, *p*-nitrophenyl- α -L-arabinofuranoside, 4-nitrophenyl-asetat, 4-nitrophenyl- α -D-galactopiranoside and *p*-nitrophenyl- β -D-glucopiranoside according to Saha (2001).

Kinetic parameters

Kinetic parameters were determined by incubating the enzyme with different amount of substrate. Xylanase was incubated with birchwood xylan (0.05-0.25%) in citrate phosphate buffer pH 5 at 90°C. The value of the Michaelis constant were determined from Lineweaver-Burk plots.

RESULTS AND DISCUSSION

Production of xylanase

The daily production curve of xylanase *Streptomyces* spp 234P-16 tested at pH 7.2 and a temperature of 37°C (Figure 1). The highest xylanase production was reached on Day-5 with the activity of 0.422 Unit/mL. The optimum time of xylanase production was then used as the standard harvest time in the next xylanase production.

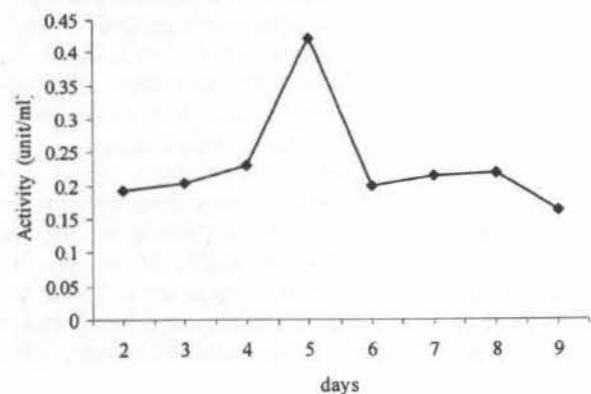
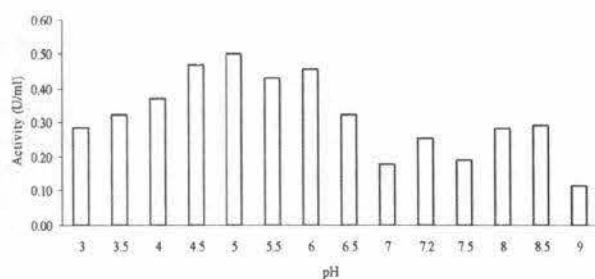


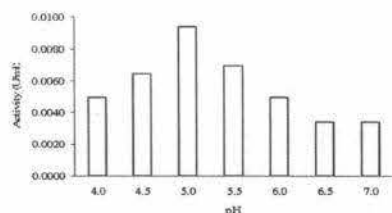
Figure 1. Production curve of *Streptomyces* sp. 234P-16 xylanase measured at 37 °C and pH 7.2.

Effect of pH on xylanase activity

The effect of pH on the crude xylanase activity measured at 37°C (A) and purified enzyme (B) has been shown in Figure 2.



A



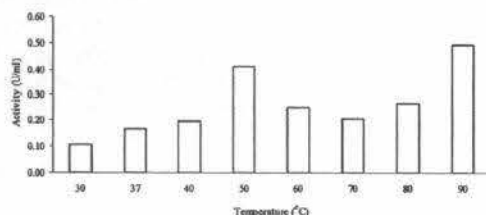
B

Figure 2. Effect of pH on *Streptomyces* sp 234P-16 crude xylanase activity measured at 37°C(A) and purified xylanase activity measured at 90°C (B).

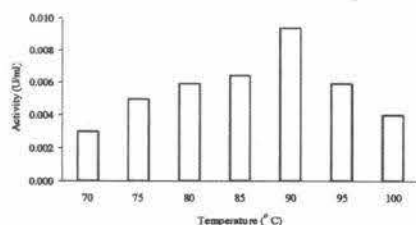
Crude and purified xylanase had its highest activity at pH 5, but the crude enzyme also demonstrated quite high activity at pH 4.5 to 6. Enzyme has specific optimum pH, which is responsible for maximum enzyme activity (Lehninger, 1982). The characteristic of enzyme's optimum pH is the condition where the catalytic site of the enzyme is at the expected ionization level (Whitaker, 1994).

Effect of temperature on xylanase activity

Figure 3 shows the effect of temperature on the activity of xylanase tested at pH 5. Xylanase displayed its optimum temperature, which was at 90°C. Temperature fluctuation can influence the integrity of secondary, tertiary and quaternary structure of enzyme that can affect on enzyme activity (Whitaker, 1994).



A



B

Figure 3. Temperature effect on *Streptomyces* sp 234P-16 crude extract (A) and purified (B) xylanase activity measured at pH 5.

Enzyme stability

At the optimum temperature, the crude enzyme have half-life time about 4 hours whereas the purified enzyme about 2.5 hours. Enzyme stability was affected by protein, carbohydrate and cations on the medium. Crude enzyme was more stable than the purified extract perhaps is due to the cations and other protein that function as an stabilizer for the enzyme. Enzyme thermostability is also due to the enzyme ability to maintain its three dimensional structure (Whitaker, 1994).

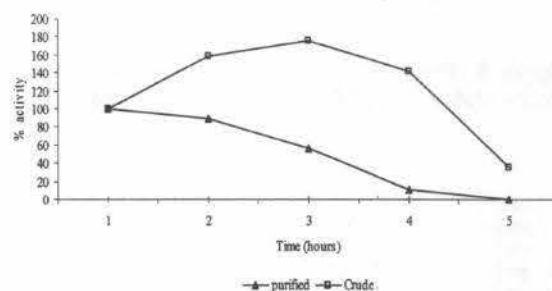


Figure 4. Stability of crude and purified xylanase *Streptomyces* sp 234P-16 on 90°C

Hydrolysis analysis using HPLC

The main product of the xylan hydrolysis was arabinose (96.61 ppm) whereas xylose just 26.55 ppm. One of the enzyme should be arabinofuranosidase.

Purification

It is clear that the best condition for precipitation xylanase was 70% acetone as can be seen in Table 2. Acetone concentration of 70% will then used for precipitation xylanase for chromatography application.

Table 2. Sedimentation of crude xylanase with acetone.

| % acetone | xylanase activity (Unit/mL) | protein content (mg/mL) | specific activity (Unit/mg) |
|--------------|-----------------------------|-------------------------|-----------------------------|
| Crude enzyme | 0.07 | 0.06 | 1.15 |
| 00-60 | 0.13 | 0.11 | 1.24 |
| 70 | 0.23 | 0.05 | 4.76 |
| 80 | 0.07 | 0.03 | 2.74 |
| 90 | 0.04 | 0.02 | 2.33 |

The result of purification using anion exchange column showed two peak for xylanase activity and protein content (fraction 15-27 and fraction 27-33). Fraction 19-23 was collected and concentrated using PEG6000 and then dialysis for 4 hours using 0.2M phosphate buffer pH 7. The purification factor increased 6x (Table 3). The concentrated enzyme was then applied to the gel filtration column.

The result of purification using gel filtration showed one peak of xylanase activity and protein content. Using gel filtration increased the purification factor to 17x (Table 3) and the SDS PAGE analysis showed one band with molecular mass of 42.5 kDa (Figure 7).

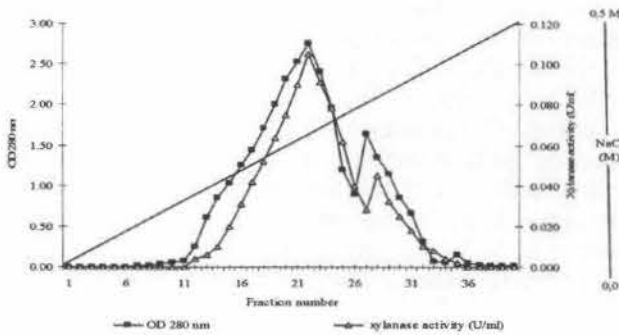


Figure 5. Profile of xylanase anion exchange chromatography elution using DEAE-Sephadex A-50 matrix.

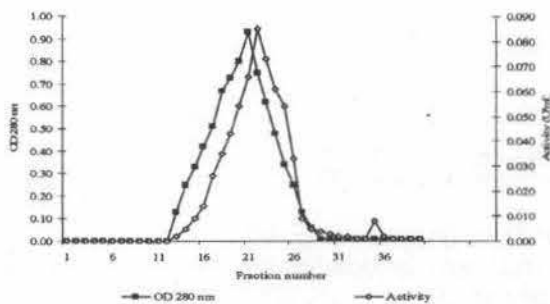


Figure 6. Profile of xylanases chromatography elution with Sephadex G100 matrix.

Table 3. Purification of *Streptomyces* sp. 234P-16 xylanase.

| Steps | Vol (mL) | Total Protein (mg) | Total Activity (Unit) | Specific Activity (U/mg) | Yield (%) | Purification factor |
|----------------|----------|--------------------|-----------------------|--------------------------|-----------|---------------------|
| Crude extract | 80 | 4.80 | 9.76 | 2.03 | 100 | 1 |
| 60-70% acetone | 20 | 0.98 | 4.66 | 4.78 | 47.75 | 2.34 |
| Anion exchange | 10 | 0.08 | 1.05 | 12.96 | 10.76 | 6.38 |
| Gel filtration | 6 | 0.01 | 0.51 | 35.42 | 5.23 | 17.42 |

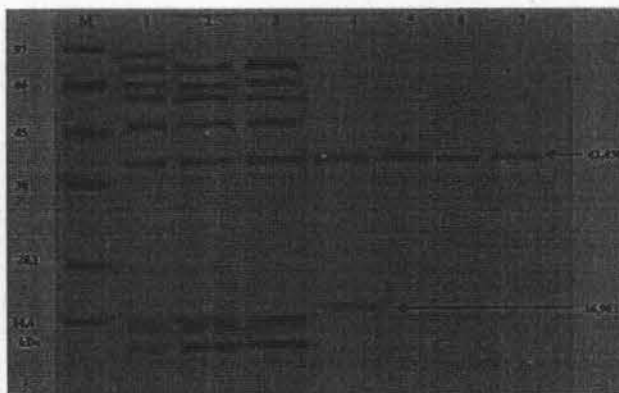


Figure 7. Profile of SDS-PAGE of the purified xylanase from: (1) crude extract, (2-3) 60-70% acetone fraction, (4) anion exchange fraction (19-23), (5-7) gel filtration fraction (21-23). (M) marker,

Substrate specificity

Assays with substrate specific showed that this enzyme has an activity on *p*-nitrophenyl- α -L-arabinofuranosidase and a β -xilosidase activity (Table 3). Activity of these two enzymes activity in one protein showed a bifunctional enzyme. This kind of enzyme was also report from *Trichoderma reesei*, *Clostridium stercoararium*, *Lycopersicon esculentum* and *Thermomonospora fusca* BD25 (Clark et al. 1996; Kaneko et al. 2000; Tuncer 2000; Itai et al. 2003).

Table 3. Substrate specificity of purified xylanase *Streptomyces* spp. 234P-16 on pH 5 and 90°C.

| Specific Substrate | Activity (U/mL) |
|--|-----------------|
| <i>p</i> -nitrophenyl- β -D-xilanopiranoside | 1.0 |
| <i>p</i> -nitrophenyl- α -D-arabinofuranoside | 40.2 |
| <i>p</i> -nitrophenyl- β -D-glucuropiranoside | 0.0 |
| <i>p</i> -nitrophenyl- α -D-galactopiranoside | 0.0 |
| 4-nitrophenyl-acetat | 0.0 |

Kinetic parameters

Reciprocal plots showed apparent K_m and V_{max} values of 1.98 mg/mL and 523 mmol/minute/mg. *Streptomyces* sp. S38 that produced one endo-xilanase 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). Several K_m dan V_{maks} value from α -L-arabinofuranosidases *Streptomyces* have also been reported: α -L-arabinofuranosidase from *Streptomyces* sp. have K_m and V_{maks} of 3,3 mg/mL and nilai 302 μ mol/minute/mg respectively (Belfaquih et al., 2002). *Streptomyces* sp. B-12-2 has a K_m value of 0,8 and 5,8 mg/mL and a V_{maks} value of 162 and 470 μ mol/minute/mg, respectively (Elegir et al.1994). Xylanase from *Streptomyces* T7 have a K_m value of 10 mg/mL and a V_{maks} value of 7610 μ mol/minute/mg (Kesker, 1992). Xylanase from *Streptomyces* sp. QG-11-3 have a K_m and V_{maks} value of 1,2 mg/mL and 158,85 μ mol/minute/mg respectively (Beg et al., 2001).

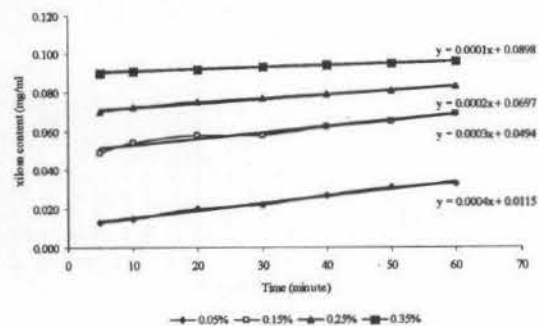


Figure 8. Xylose content on several Birchwood xylan concentration after incubation at pH 5 and 90 °C

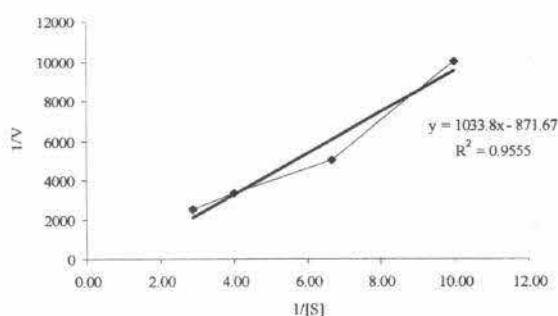


Figure 9. Correlation between $1/[S]$ and $1/[V]$

CONCLUSION

Streptomyces sp. 234P-16 was able to produce two xylanolytic enzymes α -L-arabinofuranosidase and β -xylosidase. Crude extract of xylanase was precipitated by 60-70% acetone gradually, purified with anion exchange chromatography using Sephadex A-50 and gel filtration chromatography using Sephadex G-100. The molecular mass of the purified xylanase after ion-exchange was 42.456 kDa and 16.903 kDa but gel filtration showed the molecular mass 42.456 kDa. The optimum temperature and pH was 90 °C and 5.0 respectively. The xylanase could resist of heating at 90 °C for 180 minute. The result of kinetic analysis showed that V_{max} was 523 $\mu\text{mol}/\text{minute}/\text{mg}$ and K_m was 1.98 mg/ml .

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