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Characterization of Xylanase *Streptomyces* spp. SKK1-8

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***Streptomyces* spp. SKK1-8 producing xylanase was isolated from soil sample from Sukabumi West Java. The xylanase have an optimum condition at pH 6 and 50 °C. Addition of 5 mM Cu^{2+} decreased the xylanase activity up to about 77%, whereas not by other cations. The xylanase was stable at 3 °C for 48 hours, and the enzyme half lifetime was 1 hour 45 minute at 50 °C. This xylanase showed the highest activity on oatspelt xylan, and their molecular masses were estimated approximately 16.80, 15.21, and 13.86 kDa. HPLC analysis showed that xylosa and arabinosa were the main hydrolytic product of birchwood xylan.**

Key words: xilanase, *Streptomyces* spp., characterization, zymogram and SDS-PAGE, stability

INTRODUCTION

Hemicellulose is a part of plant cell wall that composes approximately 25-30% of wood total dry weight (Perez *et al.* 2002). Hemicelluloses have a very complex structure, comprised of various types of sugar and intermonometrix (Deobald & Crawford 2002). Most part of plant cell hemicelluloses is xylan; it is the second most abundant polysaccharide available in nature after cellulose (Kubata *et al.* 1994; Saha 2002).

Xylan belongs to complex heteropolysaccharide and its backbone chain consists of units of D-xylopiranose homopolimers linked by ß-1,4 D-glycosidic bond (Saha 2002; Tseng *et al.* 2002). This chain may have several substituents constructed its side chain, such as the groups of O-acetyl, α-L-arabinofuranosyl, D-glucuronyl, and residue of O-methyl-D-glucuronyl. Hydrolysis of xylan involves two main types of enzymes, i.e. endo-β-1,4-xylanase (1,4-β-xylan xylanohydrolase) and β-xylosidase (β-D-xylosida xylohydrolase) (Kubata *et al*. 1994; Silveira *et al.* 1999; Saha 2003; Ali *et al.* 2004).

Several groups of microorganisms such as fungi, bacteria, and protozoa, were known for their ability to produce xylanase (Lin *et al.* 1999; Beg *et al.* 2001; Saha 2002; Devillard *et al.* 2003; Ryan *et al.* 2003). One of the bacteria group that is very potential in producing xylanase is Actinomycetes, especially that of *Streptomyces* (Georis *et al.* 2000; Kaneko *et al.* 2000; Wang *et al.* 2003; Kansoh & Nagieb 2004). Actinomycetes is a group of filamentous bacteria that form mycelia, have a Gram Positive nature, and mostly form spores (Madigan *et al.* 2000).

Nowadays, bioconversion of hemicellulose attains great attention due to its huge application in industry. Xylanase can be used to reduce the length of time needed for baking bread and cake. It is also able to decrease food viscosity and hence increases its digestibility, and also to make synthetic low calorie-sweetener. Xylanase is also use in pulp and paper industry, and use to reduce chlorine usage (Gilbert & Hazlewood 1993; Breccia *et al.* 1998; Kulkarni *et al.* 1999; Li *et al.* 2000; Ali *et al.* 2004).

The aim of this study was to characterize xylanase produced by *Streptomyces* spp strain SKK1-8. The strain was isolated from forest soil in Sukabumi, West Java.

METHODS

Isolates Rejuvenation and Inoculum Preparation. Isolate of *Streptomyces* SKK1-8 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 media (1% yeast extract, 10.3% sucrose, 0.5% Birchwood Xylan, 1.5% agar-agar). Incubation was performed at 30 °C for 7 days.

Determining the Optimum Time for Xylanase Production and Activity. As much as two cockbors of isolate grown in xylan media was inoculated to 100 ml production media in 500 ml Erlenmeyer. They were incubated with 140 rpm agitation at 30 °C for 10 days. The culture was centrifuged at 10.000 x g (5 minutes) everyday to obtain the xylanase crude extract. The crude extract activity was measured by using DNS (Dinitrosalisilic Acid) method by Miller (1959) with xylosa as the standard. The yielded reducing-sugar substance was assessed by spectrophotometer with a wavelength of 540 nm. One unit xylanase activity was defined as the amount of enzyme which produces 1 µmol xylosa per minute. Protein concentration (mg/ml) was defined by using Bradford (1976). *Bovine serum albumin* (BSA) was used as the protein standard.

Xylanase Characterization. Characterization of crude extract of the enzyme included the determination of optimum temperature and pH, stability, and the effect of bivalent cation. The assessment in variety of pH was carried out within pH 3.0-9.0 with 0.5 interval. The determination of optimum temperature was performed from 30 up to 90 °C with 10 °C

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interval. The stability of xylanase crude extract was tested by incubating the crude extract without substrate in two different temperatures, first at optimum temperature and second at 3 °C storage temperature. Six kinds of cations (Ca^{2+}, Zn^{2+}, Cu^{2+}, Mg^{2+}, Fe^{2+}, and Co^{2+}) derived from $CaCl_2$, $ZnCl_2$, $CuCl_2$, $MgCl_2$, $FeSO_4 \cdot 7H_2O$, and $CoCl_2$, were added separately to make final concentration of 1 mM and 5 mM to observe the effect of cation on the enzyme activity.

Determining the Enzyme Activity in Several Kinds of Xylan Substrates. The determination was defined by assessing the crude extract activity in Birchwood Xylan, Oatspelt Xylan, Wheat Arabinoxylan, Beechwood Xylan, and Carboxy Methyl Cellulose (CMC).

Sedimentation of Xylanase Crude Extract with Acetone. The xylanase crude extract was sedimented by using several percentages of acetone saturation to explore the optimum condition of xylanase fractionation by acetone. Table of acetone saturation used was based on Scopes (1987). The percentage of the saturation used was varied from 20-90%.

Analysis of Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Zymogram. Protein electrophoresis was conducted by using method of Laemmli (1970) with acrylamide concentration of 4% for collector gel and 15% for separator gel. A total of 20 µl crude extract enzyme was applied into each SDS-PAGE well. Crude extract of enzyme was resulted from sedimentation with 90% acetone. The enzyme activity was 1.379 U/ml and had 0.519 mg/ml protein concentrations. Sigma markerTM M 3913 (range from 6.5-66 kD) was used for SDS-PAGE standard. The activity of xylanase within the gel was determined by zymogram analysis, by adding 1% (w/v) of Birchwood Xylan to polyacrylamide gel before polymerization. After electrophoresis was carried out, the gel was renatured by soaking it into 2.5% (w/v) Triton-X 100 for 1 hour. The gel was then incubated for two hours in 0.2 M phosphate citrate buffer within 50 °C. Gel staining was carried out with 0.1% (w/v) congo red for 30 minutes, and gel washing was using NaCl 1 M.

Analysis of Xylanase Hydrolysis Product by High Performance Liquid Chromatography (HPLC). Xylanase produced by sedimentation process using acetone was diluted in 5 ml 0.5% (w/v) Birchwood Xylan substrate under enzyme optimum pH. Then, it was incubated at 50 °C for 5 hours. The hydrolysis product was then analysed using HPLC (Waters, USA) with the following conditions: Column: Carbohydrate, Detector: Bias Index, Solvent: 60% (v/v) Methanol, Flowing speed respectively for 1 ml/minute, Injection volume: 10-20 µl, Column temperature: 30 °C. We used xylosa and arabinosa standard (Sigma, St Louis), each concentration is 400 ppm.

RESULTS

Determining Xylanase Optimum Production Time and Activity. The daily production of xylanase was tested at pH 7.2 and a temperature of 37 °C. The highest production was reached on day-10 with the activity of 0.65 Unit/ml (Figure 1). That optimum time of production was then used as the standard harvest time in the next xylanase production.

Xylanase Characterization. The effect of pH on specific activity was measured at 37 °C (Figure 2). Xylanase had its highest activity at pH 6, but also demonstrated quite high activity at pH 3, 7.2, and 8.5.

The effect of temperature on specific activity was tested at pH 6. Xylanase *Streptomyces* spp. SKK1-8 displayed its optimum temperature at 50 °C (Figure 3).

The stability of xylanase during incubation at 50 °C differed with its stability during incubation at 3 °C (Figure 4).

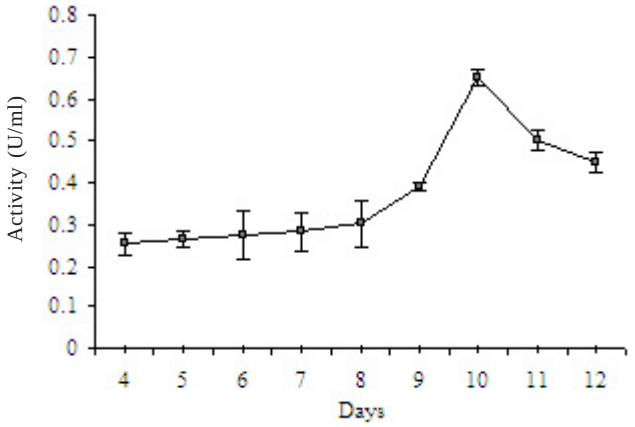

Figure 1. Production curve of *Streptomyces* spp. SKK1-8 xylanase measured on 37 °C and pH 7.2.

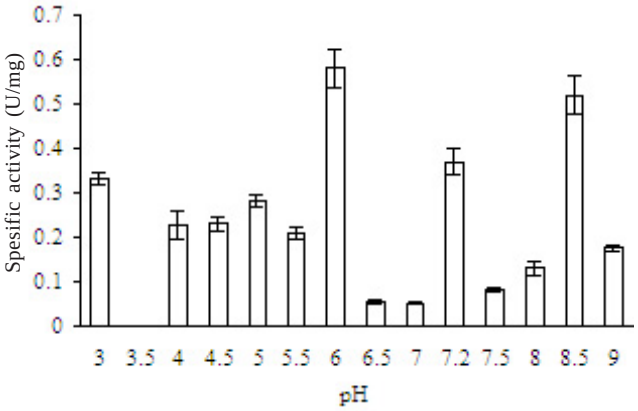

Figure 2. Effect of pH on *Streptomyces* spp. SKK 1-8 xylanase specivicity activity measured on 37 °C.

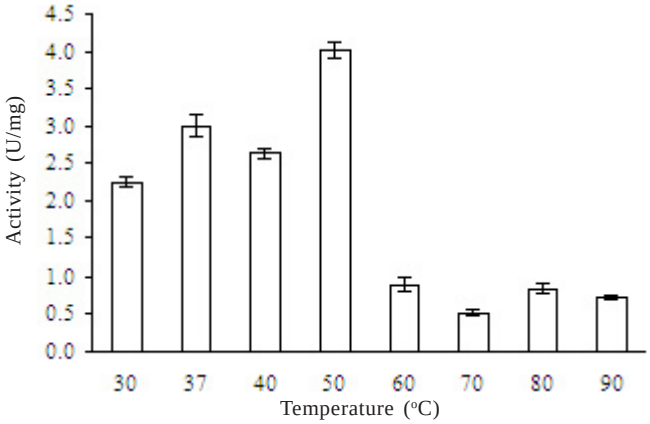

Figure 3. Temperature effect on *Streptomyces* spp. SKK 1-8 xylanase specivicity activity measured at pH 6.

At its optimum temperature there was a drastic decrease during the first 2 hours and continued to descend until the 8th hour. At the incubation temperature of 3 °C, was able to maintain its activity for 24 hours and lowered modestly after that.

The effect of cations (1 and 5 mM in concentration) on the activity of xylanase were tested 50 °C and pH 6 (Figure 5). Generally, the cations reduced the activity slightly, except 5 mM Cu^{2+} reduced 75% of the enzyme activity.

Determining Enzyme Activity in Several Kinds of Xylan Substrates. The acetone concentration that gave the highest activity was 90%. It demonstrated its highest activity in Oatspelt xylan (Figure 6).

SDS-PAGE Analysis and Zimogram. Acetone fraction of SDS-PAGE xylanase showed at least 17 protein bands (Figure 7a). This zymogram was conducted at the pH levels that gave quite high enzyme activity levels with the incubation temperature of 50 °C. Gel incubation treatment at pH 3 and 6 showed three active protein bands with the relative molecule mass of 16.80, 15.21, and 13.86 kDa. Through the gel incubation treatment at pH 7, there were two active protein bands with relative molecule mass of 16.80 and 15.21 kDa, while gel incubation at pH 8.5 only showed one active protein band with relative molecule mass of 15.21 kDa (Figure 7b).

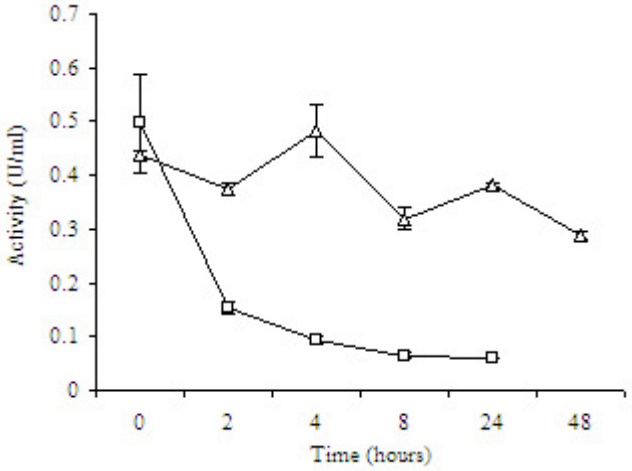

Figure 4. Stability curve of *Streptomyces* spp. SKK1-8 at 3 and 50 °C.

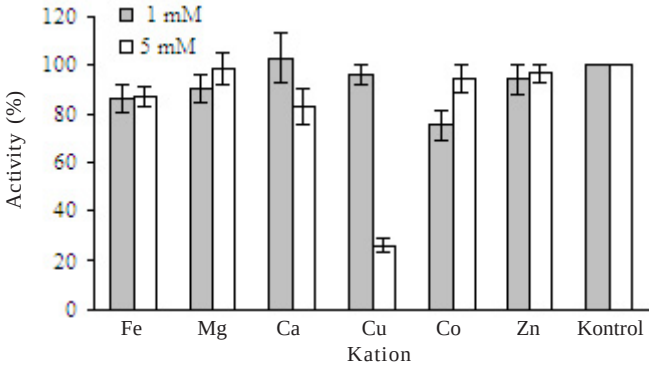

Figure 5. Kation effects on *Streptomyces* spp. SKK 1-8 xylanase measured at pH 6 and 50 °C.

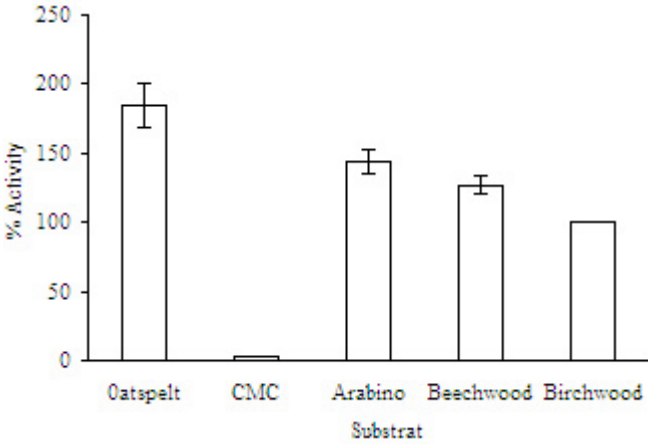

Figure 6. Activity of *Streptomyces* spp. SKK 1-8 xylanase on several substrat measured at pH 6 and 50 °C.

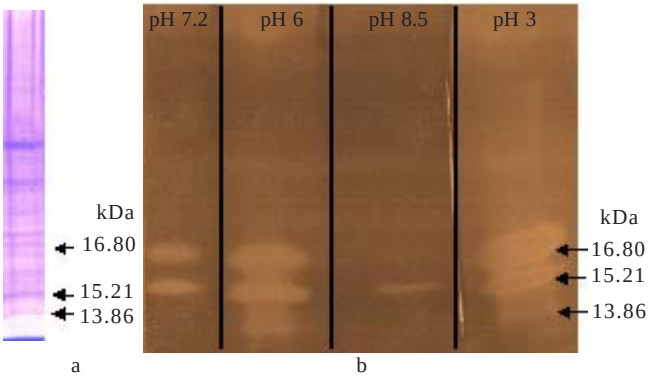

Figure 7. a. SDS PAGE crude xilanase from *Streptomyces* spp. SKK 1-8. b. Zymogram from *Streptomyces* spp. SKK1-8 xylanase on several pH.

Analyzing the Hydrolysis Product of Birchwood Xylan using HPLC. The hydrolysis products of 0.5% (b/v) Birchwood Xylan by xylanase *Streptomyces* spp. SKK1-8 were xylosa and arabinosa with concentration of 282.74 and 119.10 ppm, respectively.

DISCUSSION

Enzyme has specific optimum pH; that is, the pH produce the maximum enzyme activity (Lehninger 1982). Xylanase *Streptomyces* spp. SKK1-8 had an optimum condition at pH 6 and temperature of 50 °C. Hence, it can be categorized as acidophilic enzyme and high optimum temperature (thermophyl). The characteristic of enzyme's optimum pH is the condition where the catalytic site of the enzyme is at the expected ionization level. Since chemical reaction is strongly effected by temperature, the reaction catalyzed by enzyme is also sensitive to temperature. Temperature fluctuation can affect the integrity of the secondary, tertiary, and quaternary structure of enzyme protein, which then will affect the enzymatic activity (Whitaker 1994).

Half-life time of xylanase *Streptomyces* spp. SKK1-8 at 50 °C was 1 hour 45 minutes. An enzyme's half-life time can be used to determine its stability. Xylanase *Streptomyces* spp.

SKK1-8 that has optimum temperature at 60 °C could still maintain 85% of its activity at 50 °C after 24 hours of incubation (Beg *et al.* 2001). Xylanase *S. lividans* 66 loses all its activities when incubated at 60 °C for 2 hours (Kluepfel *et al.* 1992), while xylanase *Humicola grisea* var *thermoida* is stable for 1 hour when incubated at 50 °C (Almeida *et al.* 1995).

The decrease in xylanase activity along with the incubation period at optimum temperature is believed to be related to the change in the three-dimensional structure of the enzyme protein due to heating. Enzyme stability at 3 °C is caused by the enzyme's inactive or static condition (Whitaker 1994).

The activity of xylanase *Streptomyces* spp. SKK1-8 is hindered by cation Cu^{2+} in concentration of 5 mM. Kluepfel *et al*. (1992) and Almeida *et al.* (1995) reported the same result for *S. lividans* 66 and to *H. grisea* var *thermoida*, respectively.

Xylanase produced by *Streptomyces* spp. SKK1-8 incubated at variety of pHs demonstrated various active bands on the zymogram. It showed the activity at a wide range of pH. Xylanase with molecule weight of 15.21 kDa was found at every gel incubation treatment having different range of pH. Other protein bands (16.8 and 13.8 kDa) that were found only at certain pH showed that every enzyme has its own specific optimum pH. Generally, enzyme will be denatured at a very low or high pH (Whitaker 1994). The more number of active bands emerge at acid range of pH indicates that xylanase *Streptomyces* spp. SKK1-8 tends to be acidophilic in nature.

Based on the enzyme protein molecule weight, xylanase *Streptomyces* spp. SKK1-8 belongs to xylanase group with relatively low molecule mass (< 30 kDa). Wang *et al*. (2003) reported that relative molecule weight of *S. actuosus* A-151 is at the range of 21-34 kDa, and three xylanase *S. lividans* have relative molecule weights of 22, 31, and 45 kDa, respectively (Shareck *et al*. used as a reference in Kaneko *et al.* 2000). One of the three xylanase produced by *Streptomyces* sp. Strain S38 has low molecule mass (Georis *et al*. 2000).

Xylan from several sources like grass, cereal, soft wood, and hard wood have differences in terms of their monomer component. Birchwood xylan is made up of 89.3% xylose, 1% arabinose, and 8.3% anhydrouronate acid. Wheat arabinoxylan consists of 65.8% xylose, 33.5% arabinose, 0.3% glucose, 0.1% manose, 0.1% galactose (Saha 1003). The highest activity unit of xylanase *Streptomyces* spp. SKK1-8 was shown at the oatspelt xylan substrate. Similar result was obtain for *Streptomyces* sp. Strain S38 (Georis *et al*. 2000), *S. malaysiensis* (Nascinento *et al.* 2003) and *S. halstedii* JM8 (Ruiz-Arribas *et al*. 1995). According to Li *et al*. (2000), the difference in the xylosa chain length and the number of the side-chains at xylan substrate affect the xylanase performance in hydrolyzing the xylan structure. The shorter the xylosa chain, the slower the xylanase hydrolytic activity. This is due to the decrease of xylanase's bounding site over its substrate.

Xylanase *Streptomyces* spp. SKK1-8 demonstrated very low activity in the substrate of carboxy methyl cellulose (CMC). Xylanase examination in CMC substrate needs to be conducted because there are several xylanase that is able to hydrolyze not only xylan but also cellulose (Tjusibo *et al.* 1992). The low activity in CMC media indicated the absence of enzymatic activity over cellulose structure. It occurs due to the difference in structure and composition of monomers between cellulose and xylan. The absence of cellulolitic activity of xylanase provides practical application in pulp industry.

The production of xylosa out of Birchwood Xylan hydrolysis indicated the activity of β-xylosidase that liberated xylosa from the tip of non-reducing xylan's backbone. Meanwhile, arabinosa production indicated the existence of α-L-arabinofuranosidase that increases arabinoxylan saccharification (Ryan *et al*. 2003). Xylanase hydrolysis product are commonly induced to produce oligosaccharide as reported on *S. lividans* 66 (Kluepfel *et al*. 1992), *H. grisea* var thermoida (Almeida *et al.* 1995), *Sulfolobus solfataricus* (Cannino *et al*. 2004), and *Thermotoga maritima* (Xue & Shao 2004). However, xylanase of *Bacillus* strain SPS-0 (Batallon *et al*. 2000) was reported to give xylosa as hydrolysis product.

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