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Endo-xylanase enzyme from marine actinomycetes and its potential for xylooligosaccharide production

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Abstract. Marine microorganism was emerging as the great source for the discovery of novel enzyme. The search and discovery of new enzymes derived from actinomycetes have received much attention nowadays due to a growing need for applications in various industries including food industry. Endo- β -1,4-xylanases is considered one of critical enzyme involves in the degradation of xylan that is able to cleave the β -1,4-glycosidic linkages in the xylan backbone resulting in various xylooligosaccharides and xylose. Screening and characterization of novel actinomycetes that is capable of producing high xylanase are necessary to produce xylooligosaccharides from hemicellulose hydrolysis process. The objective of this research is screening and characterizing of novel marine actinomycetes from working culture of Research Center for Biotechnology, Biotechnology Culture Collection (BTCC) that are capable of producing high-level hemicellulase-degrading enzymes, especially endo-xylanase enzyme. Approximately, 70 strains from Indonesia marine Actinomycetes have been screened by using congo red, SDS-PAGE and zymogram methods. Of these 70 strains, 3 strains were successfully identified that is capable in producing high level of xylanase enzymes based on the diameter of clear zone more than 1.5 cm on xylan plate medium pH 5.0 and 6.0. Three strains from marine Actinomycetes were identified based on analysis a 16S rRNA gene sequence revealed that all of strains belonging to the genus *Streptomyces*. Strain 47 is closely related with *Streptomyces variabilis* (98%). All strains had ability to produce xylanase at optimum pH 5.0 and temperature range between 50- 60 °C with range activity from 2.5 – 4.3 U/mL. Each isolate had a molecular weight from 20-50 kDa based on SDS-PAGE and zymogram analysis. Based on TLC analysis using beechwood xylan, each isolate had ability to produce xylooligosaccharides. These characterizations showed that these isolates are potentially used for xylooligosaccharides productions. Strain 47 was selected for the next analysis for molecular cloning based on the pattern and results of TLC clearer than strain 41 and strain 42.

Keywords: Endo-xylanase, marine actinomycetes, *Streptomyces*, xylooligosaccharide

1. Introduction

Hemicellulose is the second most abundant renewable polymer saccharide in nature after cellulose. Hemicellulose plays a role in crosslinking the other components of biomass via hydrogen and covalent



bonds [1] [2] [3]. The hemicellulosic system is more complex due to the heterologous nature of hemicellulose, which is composed of various sugar units, with attached side chains and side groups. The hemicellulosic fraction represents a rich source of xylooligosaccharides (XOS) and D-xylose, a biotechnologically-relevant sugar for bioethanol production by C5-fermenting microorganisms and as precursor for synthesis of chemical compounds such as xylitol and organic acids, which are value-added products for industry. There is growing interest in production of XOS based on their beneficial health effects including prebiotic effects in gastroenteritis, functional properties as a soluble fiber and ability to reduce cholesterol blood levels [4]. It can be used as food ingredients, symbiotic food, pharmaceuticals or fertilizer [5].

β -1,4-xylan is a major component of hemicellulose and has a backbone of β -1,4-linked D-xylopyranosidase residues substituted with acetyl, arabinosyl and uronyl side chains [6]. Xylanolytic enzyme system carrying out complete hydrolysis of xylan requires the synergistic action of different xylanolytic enzymes, including endo-1,4- β -D-xylanases (EC 3.2.1.8), exo-1,4- β -D-xylosidases (EC 3.2.1.37) and accessory enzymes, such as α -glucuronidases (EC 3.2.1.139), acetyl xylan esterases (EC 3.1.1.6), α -L-arabinofuranosidase (EC 3.2.1.55), α -galactosidase (EC 3.2.1.22) and phenolic acid esterase (ferulic and p-caumaric acid). All these enzymes act cooperatively to convert xylan into its constituent sugars.

Marine microorganisms are widely distributed in ocean all over the world including Indonesia. Indonesia consists of three-quarter of sea containing high biological diversity, including microorganism. The ocean floor has been recently demonstrated as an ecosystem with many unique forms of actinomycetes [7] [8]. It appears that they are widely distributed throughout the ocean and can be found in seawater [9] [10]. The presence of such a multifunctional xylanolytic enzyme system is quite widespread among the fungi, actinomycetes, and bacteria. *Streptomyces* sp. has been reported as the dominant xylanolytic species of actinomycetes producing the hemicellulose degradation enzymes [11]. *Streptomyces* sp. is model organisms for the studies of several enzymes important in the food industry and other industries. A strategy of screening and characterization of novel actinomycetes capable of producing high-level xylanase is necessary. Characterization of novel bacteria capable of producing high-level xylanase seems to be all suited starting point for obtaining improved hemicellulose hydrolysis to produce xylooligosaccharides. The purpose of this study was to screen and characterizing of novel marine actinomycetes from working culture of Research Center for Biotechnology, Biotechnology Culture Collection (BTCC) that is capable of producing high-level hemicellulase-degrading enzymes, especially for endo-xylanase enzyme and study potential the selected strains for xylooligosaccharide production.

2. Materials and Methods

2.1. Biomass preparation

The sugarcane bagasse used in this study was obtained from a sugarcane factory, PG Rajawali, at Subang, West Java, Indonesia. The sugarcane bagasse was sun-dried, ground, and sieved for material with particle sizes equal to a 40-60 mesh. The material was kept in sealed plastic bags that were stored in a container.

2.2. Strain, Materials, and Chemicals

The actinomycetes used in this study were obtained from working culture of Research Center for Biotechnology, Biotechnology Culture Collection (BTCC), Indonesian Institute of Sciences (LIPI). Xylan was purchased from Sigma-Aldrich (St. Louis, Mo. USA) and Nacalai, Japan. All other reagents were of the highest grade available.

2.3. Culture and growth conditions

Actinomycetes were grown at 28 °C in 4 mL ISP2 medium containing artificial seawater (ASW) for 3 days. The composition of the ISP2 medium (w/v) was as follows: Yeast extract 0.4 %, Malt extract 1%, Glucose 0.4%. Erlenmeyer (100 mL) containing 10 mL ISP2 medium without glucose, plus 0.5 % of sugar cane bagasse were sterilized, cooled and inoculated with pre-culture of the isolate. The

fermentation was carried out on a rotary shaker at 28 °C for 7 days. Sampling was carried out every 24 hours, and the enzyme was harvested for the recovery of xylanase enzyme from the culture supernatant by centrifuge in 5000 rpm, 4 °C for 10 min. Xylanase degrading activity was conducted on xylan agar, pH 5 and 6. Clear zones were appeared around growing bacterial colonies indicating xylanase hydrolysis.

2.4. Screening for lignocelluloses degrading enzyme

Xylanase degrading activity was conducted on xylan agar. Clear zones were appeared around growing bacterial colonies indicating xylan hydrolysis. The isolates which exhibited maximal zone of degradation (> 1.5 cm) were chosen as the organism for the further study. Strains were cultured on ISP2 agar medium. A single colony was pre-cultured in ISP2 broth at 28 °C, 190 rpm for 3 days, then inoculated into 10 mL modified ISP2 broth contained 0.4% yeast extract, 1.0% malt extract, and 0.5% each of sugarcane bagasse in 100 mL Erlenmeyer flasks. The fermentation was carried out on a rotary shaker at 28 °C and 190 rpm for 7 days. Sampling was carried out every 24 h. The xylanase was harvested from the culture supernatant via centrifugation at 4 °C and 9,000 g for 10 min. The analysis of xylanase activity against each of the xylan substrates was measured on xylan agar (0.5% xylan and 1.8% agar) by individually spotting an aliquot of supernatant (5 µL) taken from the culture. The plates were then incubated at 37 °C for 3 days to ensure that a clear halo formation had occurred due to the degradation of xylan by the respective enzymes. To visualize the hydrolysis zones, the plates were flooded with an aqueous solution of 0.25% Congo red (Nacalai, Kyoto, Japan) for 30 min. Then, the plates were washed with 1M NaCl for 15 min twice, and 0.5% acetic acid to more clearly check the xylan degradation. Xylan degradation appeared as clear zones around the colonies as opposed to the unclear zones that had not degraded.

2.5. Dye staining of xylan agar

The xylanolysis basal medium pH 5, 6 containing substrate xylan 0.5% and 1.8% agar were prepared and autoclaved. The sterile media was aseptically transferred to the sterile Petri dishes and inoculated with the supernatant individually (5 µL). The plate was then incubated at 37 °C for 3 days. To visualize the hydrolysis zone, the plates were flooded with an aqueous solution of 0.25% congo red for 1 hour, washed with 1M NaCl 30 min 2 times and washed with 0.5% acetic acid to get better picture. Xylan degradation around the colonies appeared as clear zone against not clear zone upgraded. To indicate the activity of organisms, diameters of clear zone around the colonies on cellulose agar plate were measured. Colonies with the highest clear zone on the plate were selected as a potential strain and subjected to saccharification test.

2.6. Determination of molecular weight by SDS-PAGE and zymogram analysis

Samples were solubilized in sample buffer, and equal amount of protein was loaded into 12% SDS-PAGE and zymogram was carried out at constant current (100 mA) for 3.5 hours [12].

2.7. Culture of lignocelluloses producing actinomycetes

Selected isolates were cultured on Erlenmeyer flask (500 mL) containing 2.5 % of sugarcane bagasse. The fermentation was carried out in 500 mL Erlenmeyer flasks on a rotary shaker at 28 °C for 7 days. At the end of the 7 days, the enzyme was harvested for the recovery of lignocellulose enzyme from the culture supernatant by centrifuge in 9000 g, 4 °C for 10 min.

2.8. Characterization of endoxylanase from the isolated candidate

Xylanase activity was assayed using 0.5% (wt/vol) birchwood glucuronoxylan (Sigma) as a substrate according to the method of [13]. The amount of reducing sugars liberated was determined using the dinitrosalicylic acid (DNS) method with xylose used as standard [14]. To determine the optimal pH of xylanase activity, evaluations were conducted between pH 3.0 and 10 under standard assay conditions using the following buffers: 50 mM sodium citrate buffer (pH 3.0-5.0), sodium phosphate buffer (pH 6.0-7.0), and Glycine-NaOH buffer (pH 7.0-10.0), respectively. The optimal temperature of the xylanase activity was evaluated by incubating the enzyme samples with the substrate at temperatures ranging from 30 to 90 °C in 50 mM sodium citrate buffer (pH 5.0).

2.9. Enzymatic hydrolysis of xylan by selected strains

The main product hydrolysis of 0.5% (w/w) xylan beechwood substrates from selected strain were carried at 45 °C in 50 mM MES buffer (pH 6.5), for various reaction time (0, 1, 2, 3, 4 and 24 h). The reaction mixture was incubated for 0, 1, 2, 3, 4 and 24 h in a shaker incubator at 30 °C. At various reaction times (0, 1, 2, 3, 4 and 24 h), the reaction mixture was withdrawn and heated at 100 °C for 5 min to terminate the reaction. The reaction mixture was spotted on TLC Silica gel 60F₂₅₄, 20-20 cm (EMD/Merck, Darmstadt, Germany) and developed with a mixture of n-Butanol/Acetic Acid/water (2:1.1:1, v/v/v). Spots were staining by using DAP that contained diphenylamine, aniline, acetone, and phosphoric acid (Merck KGaA, Darmstadt, Germany), and subsequently heating at 120 °C for 15 min. Xylose (X1), xylobiose (X2), xylotriose (X3), xylotetraose (X4) and xylopentaose (X5) and xylosehexaose (X6) from Megazyme were used as a standard.

2.10. Molecular identification of the selected strain

Molecular identification of the selected strain was conducted based on the 16S rRNA gene as established by Lisdiyanti et al. [15]. A pair of 9F (5' AGRGTTTGATCMTGGCTCAG 3') and 1510R (5' TACGGYTACCTTGTTAYGACTT 3') primers were used for amplified the 16S rRNA gene via polymerase chain reaction (PCR) technique [16]. The sequence was confirmed via ABI 3130 DNA sequencer (Applied Biosystems, Foster City, CA, USA) and then compared with others available in the GenBank/DDBJ/EMBL database.

3. Results and Discussion

3.1. Screening for endoxylanase-producing strain using agar plates

Marine microorganism is not only becoming an attractive source of novel natural products but also serving as the source of valuable genes and useful industrial enzymes [17]. We have already screened marine actinomycetes in order to determine the xylanase production capacity from 70 Indonesia Actinomycetes strains, working culture of Research Center for Biotechnology, Biotechnology Culture Collection. These strains were cultivation in ISP2 medium containing ASW using sugarcane bagasse as a carbon source. The qualitative analysis for xylanolytic activity was conducted using Congo red dye. After three days of incubation on agar plate medium buffer sodium acetate pH 5.0 and sodium phosphate pH 6.0 containing xylan as the sole carbon source, the diameters of the colonies and the clear halos around them were measured. We found three selected strains have ability to produce xylanase activity with diameter clear zone more than 1.5 cm (Figure 1).

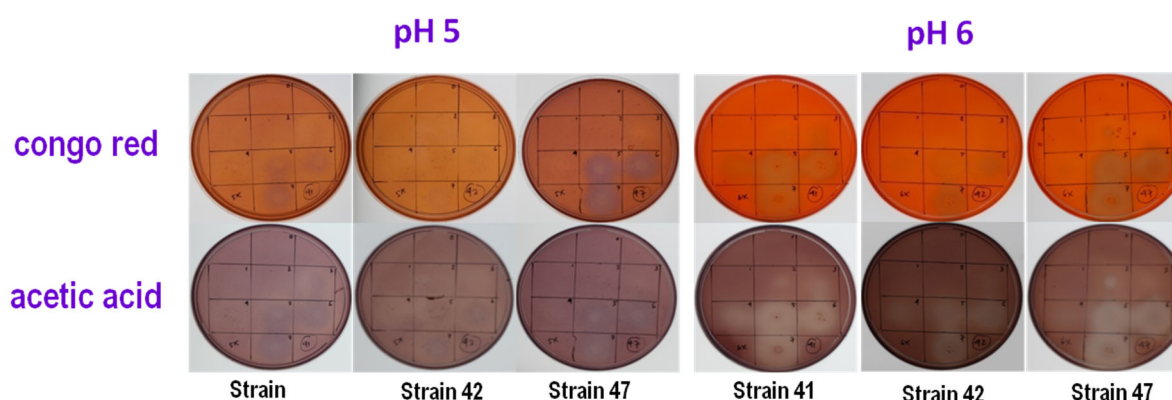


Figure 1. Clear zone in the xylan agar medium at pH 5.0 and 6.0 using the culture supernatant of the candidate strain, staining by congo red and acetic acid indicating the hydrolysis of xylanase (0, 1, 2, 3, 4, 5, 6 and 7 days cultivation).

We continue SDS-PAGE and zymogram analysis of three selected strains on xylan pH 5.0 and pH 6.0. The result of Figure 2 showed that there are four patterns of variance molecular weight range from 20-50 kDa. Strain 41 and strain 47 have similar pattern on zymogram analysis produce three thick bands predicted below 37 kDa, and one thin band predicted 50 kDa, whereas strain number 42 produces three thick bands predicted below 37 kDa.

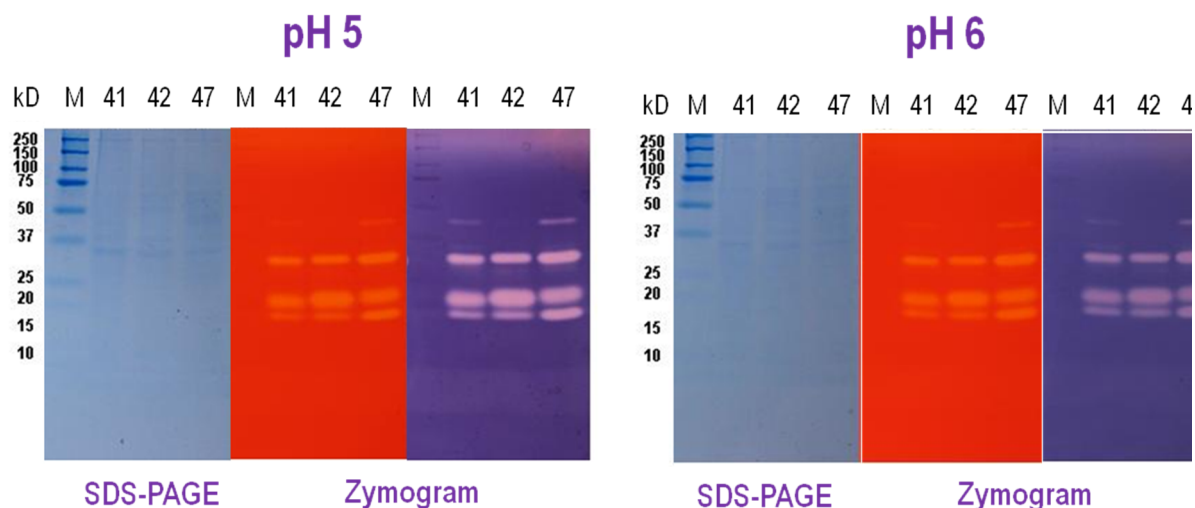


Figure 2. SDS-PAGE and zymogram analysis on xylan pH 0.05 mM sodium acetate buffer 5.0 and 0.05 mM sodium phosphate buffer 6.0 from selected strains.

3.2. Characterization of selected strains as an endoxylanase producer

Streptomyces have been found to be the most abundant hemicellulases producer among actinomycetes [18]. Several *Streptomyces* species, which are high activity as a xylanase producer have been reported from terrestrial Actinomycetes [19]. In this study, three strains from marine Actinomycetes were identified based on analysis a 16S rRNA gene sequence revealed that all of strains belonging to the genus *Streptomyces* (Table 1). Strain 47 is closely related with *Streptomyces variabilis* (98%).

The characteristic of xylanase activity was measured on various pH and temperature. The result xylanase activity on various pH was shown in Table 1. All strains were optimum at pH 5, temperature optimum range at 50 °C for strain 41 and 42 and 60 °C for strain 47 with range activity from 2.5 – 4.3 U/mL. This research has been similar to Beg et al. [20] who reported that xylanase from *Streptomyces* generally has optimal activity at 50-60 °C. Compared with other of xylanase, these xylanases were similar with xylanase from *S. megasporus* DSM 41476 which is optimum at pH 5.5 [19].

Table 1. Characterization of selected strains as a xylanase producer

Strain codes	16S rDNA analysis	pH optimum	Temperature optimum (°C)	Enzyme activity (U/mL)
Strain 41	<i>Streptomyces</i> sp.	5.0	50	2.7
Strain 42	<i>Streptomyces</i> sp.	5.0	50	2.5
Strain 47	<i>Streptomyces variabilis</i>	5.0	60	4.3

3.3. Hydrolysis of xylan commercial by selected strains

The hydrolysis of xylan substrates by the culture supernatant from three selected strains using xylan commercial was analyzed via TLC. Hydrolysis of 1% (w/v) beechwood xylan was performed containing 50 mM sodium citrate buffer (pH 5.0) in 20 mL total reaction. Each strain has ability to degrade substrate xylan to produce various xylooligosaccharide. The main hydrolysis xylooligosaccharides released using the action of endoxylanase from three strain have similar patterns from xylobiose (X2) to xylohexaose

(X6). This suggested that the xylanase from these strains belong to members of xylanase family. Kluepfel et al. [21], Yan et al. [22], Li et al. [23], Shin et al. [24] reported that many members of xylanase family 11 hydrolyze xylans to xylo-oligosaccharides with the higher degree of polymerization ≥ 2 . This suggested that the enzyme preferentially cleave the internal glycosidic bonds of these xylo-oligosaccharides. Strain 47 was selected for the next analysis for molecular cloning based on the pattern and results of TLC clearer than strain 41 and strain 42.

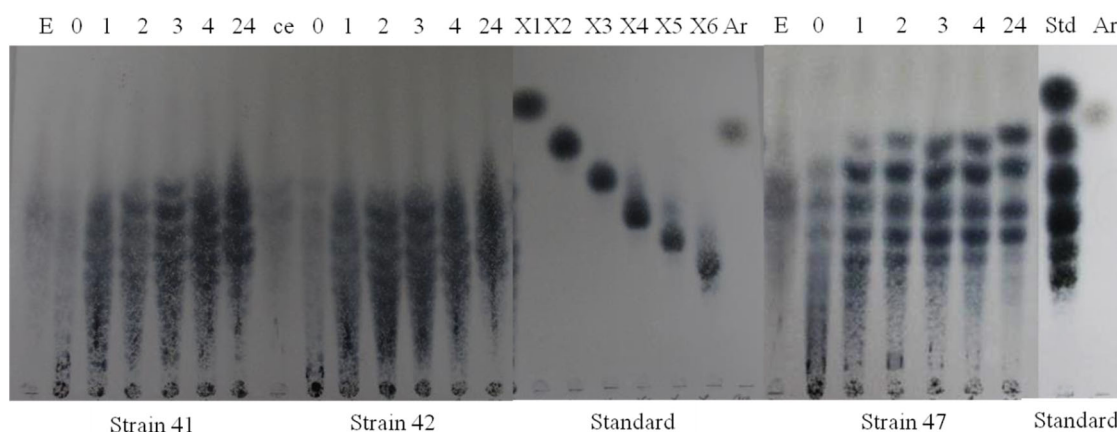


Figure 3. TLC analysis of hydrolysis products performed by three selected strains using beechwood xylan substrates. Standards (STD) : Xylose (X1), xylobiose (X2), xylotriose (X3), xylotetraose (X4) and xylopentaose (X5) and xylosehexaose (X6). Substrate concentrations 1% beechwood xylan in 50 mM sodium citrate buffer (pH 5.0), reaction time: 0, 1, 2, 3, 4, and 24 h at 30 °C, respectively.

4. Conclusion

This study report three strains were successfully identified that is capable in producing high level of xylanase enzymes based on the diameter of clear zone more than 1.5 cm on xylan plate medium pH 5.0 and 6.0. Analysis a 16S rRNA gene sequence revealed that all of strains belonging to the genus *Streptomyces*. All strains had ability to produce xylanase at optimum pH 5.0 and temperature range between 50- 60 °C with range activity from 2.5 – 4.3 U/mL. Each isolate had a molecular weight from 20-50 kDa based on SDS-PAGE and zymogram analysis. Based on TLC analysis using beechwood xylan, each isolate had ability to produce xylooligosaccharides. These characterizations showed that these isolates are potentially used for xylooligosaccharides productions. Strain 47 which is closely related with *Streptomyces variabilis* (98%) was selected for the next analysis for molecular cloning based on the pattern and results of TLC clearer than strain 41 and strain 42.

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