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Lytic polysaccharide monooxygenase of soil actinomycete with potential use for lignocellulose biodegradation

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ABSTRACT

Aims: Lytic polysaccharide monooxygenase (LPMO) is an enzyme capable of cleaving glycoside bonds of recalcitrant polysaccharides through an oxidative mechanism. LPMO activity, in synergy with hydrolytic enzymes, increases the production of monomer sugars from the biodegradation of lignocellulose. This study was aimed at evaluating actinomycete S2 strain LPMO activity based on the release of xylose as one of reducing sugar and hydrogen peroxide (H_2O_2) in the course of lignocellulosic biodegradation.
Methodology and results: The oxidation activity of LPMO from actinomycete S2 strain was measured by using the substrate of Avicel supplemented with ascorbic acid and copper ions (Cu^{2+}) to identify its effect on the release of xylose as one of reducing sugar. The optimum incubation time for the LPMO production was also conducted. Further, H_2O_2 quantitative analysis was performed as by-product of LPMO activity and 16S rRNA gene sequence of actinomycete S2 strain were subsequently determined. We found that supplementation of 1 mM ascorbic acid and 0.2 mM Cu^{2+} increased xylose as one of reducing sugar production by up to 5-fold from 255.03 to 1290 $\mu g.mL^{-1}$ after an optimal incubation period of 6 days. Based on H_2O_2 production, the LPMO activity of actinomycete S2 strain was 0.019 ± 0.001 $U.mL^{-1}$. There is likelihood that LPMO activity derived from actinomycete S2 strain has a synergistic effect with the activity of other lignocellulose-degrading enzymes. This actinomycete showed 99% similarity to the 16S rRNA gene sequence of *Streptomyces avermitilis* strain EAAG80.
Conclusion, significance, and impact of study: LPMO enzyme activity from actinomycete S2 strain as determined by the production of reducing sugar and H_2O_2 was greatly increased by supplementation with ascorbic acid as an electron donor and Cu^{2+} ions. To the best of our knowledge, this is the first elucidation of LPMO activity from an indigenous Indonesian actinomycete.

Keywords: Lignocellulose, LPMO, actinomycete, reducing sugar, *Streptomyces avermitilis*

INTRODUCTION

Lignocellulose is a major component of the cell wall of plants and is therefore very abundant on the earth. It consists of three main polymers, namely lignin (10–25%), hemicellulose (20–35%), and cellulose (35–50%) (Pradeep *et al*., 2013). Cellulose, the main component of lignocellulose, is a homogeneous linear polymer of glucosyl units with β (1→4) bonds forming microfibrils in crystalline and amorphous structures. Owing to its particular structure, it is resistant to many lignocellulose-degrading hydrolytic enzymes that are blocked from reaching their substrate (Horn *et al*., 2012).

Lytic polysaccharide monooxygenase (LPMO) is an enzyme that can break down crystalline polysaccharides, components in cellulose that are not otherwise easily accessible to lignocellulose-degrading hydrolytic enzymes such as cellulase and xylanase. Hence, LPMO enzyme can be used in the pre-treatment process of lignocellulosic waste at an early stage of biodegradation.

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LPMO acts by oxidative cleavage between β (1→4)-glucosyl units in cellulose, hemicellulose, glucan, and cellodextrin by forming oxidized gluco-oligosaccharides. Improved accessibility of lignocellulose-degrading enzymes to their substrates by LPMO is reflected in the release of reducing sugars. For example, LPMO from *Myceliophthora thermophila* (*Mt*LPMO9A) added to cellulase increases the release of glucose from cellulose (Frommhagen *et al*., 2015). Besides cellulase, another enzyme that participates in the degradation of lignocellulose is xylanase which breaks down xylan in the production of xylitol, ethanol, and oligosaccharides. Xylanase has important roles in the animal feed industry, pulp processing and paper bleaching (Patel and Savanth, 2015). Therefore, xylanase as one of the lignocellulose-degrading hydrolytic enzymes also serves as an indirect indicator of LPMO activity. In this connection, the production of reducing sugar including xylose and others catalyzed by xylanase and other hydrolytic enzymes is enhanced by LPMO activity.

It is of note that LPMO is copper-dependent enzyme, and its activity requires oxygen as well as an electron donor, such as ascorbic acid, gallic acid, or as the result of cellobiose dehydrogenase activity (Westereng *et al.*, 2013; Isaksen *et al*., 2014; Frommhagen *et al*., 2015). Accordingly, *Mt*LPMO9A activity on Avicel medium was reportedly capable of producing both oxidized oligosaccharides and xylo-oligosaccharides when ascorbic acid was supplemented as an electron donor (Frommhagen *et al*., 2015). The activity of LPMO can also be detected quantitatively based on the production of hydrogen peroxide (H_2O_2), a by-product of the futile side reaction of LPMO activity when an electron donor is available, but cellulose substrate is not. Additionally, the formation of H_2O_2 is directly proportional to the concentration of the LPMO, which itself is dependent on availability of a reductant. In that reaction, ascorbic acid or cellobiose dehydrogenase reduces the LPMO with its copper (Cu) center that activates the oxygen molecule by reducing one electron. The release of H_2O_2 can be detected through conversion of Amplex Red to resorufin using horseradish peroxidase (HRP) in a fluorometric assay (Kittl *et al*., 2012).

This research was aimed at examining LPMO activity of actinomycete S2 strain based on the increase in xylose as one of reducing sugar and the measurement of H_2O_2 production. Xylose production would strongly indicate an increase in the accessibility of xylanase in lignocellulose biodegradation. Therefore, the ability to degrade Avicel (crystalline cellulose) and xylanase activity can serve as an initial step in the screening of actinomycetes for LPMO activity. Actinomycetes are Gram-positive bacteria that have the ability for filamentous growth which facilitates these microbes in the penetration of plant tissues, thus enhancing their efficiency in the degradation of natural biopolymers (Barka *et al*., 2016). In this study, we used actinomycete S2 strain previously isolated from soil in an oil palm plantation in Jambi, Indonesia. This isolate had the best cellulase (index of 2.6) and xylanase (index of 3.87) activities as compared to other isolates with indices between 1.7 to 2.5 and 0.70 to 3.57 for cellulase and xylanase, respectively (Utarti *et al*., 2020). We initiated this study on the expectation that actinomycete S2 strain could produce LPMO enzyme that plays a synergistic role in conjunction with lignocellulose-degrading hydrolytic enzymes.

MATERIALS AND METHODS

Actinomycete isolate and culture medium composition

Actinomycete S2 strain used in this study was previously isolated from soil in an oil palm plantation in Jambi, Indonesia (Utarti *et al*., 2020). Screening for avicelase activity was conducted using Avicel medium containing 0.5 g Avicel, 0.5 g yeast extract, 0.5 g bacto peptone, 0.05 g $MgSO_4 \cdot 7H_2O$, 0.05 g $K_2HPO_4 \cdot 3H_2O$, 0.075 g KNO_3, 2 g agar powder in 100 mL of distilled water, and adjusted to pH 6.2. Xylanase activity was determined in the same medium of similar composition, but with Avicel substituted by 0.5 g beechwood xylan. Both xylanase and LPMO production was undertaken in a medium modified from Kholis *et al*., (2015). The modified medium contained 0.5 g yeast extract, 0.5 g bacto peptone, 0.05 g $MgSO_4 \cdot 7H_2O$, 0.05 g $K_2HPO_4 \cdot 3H_2O$, 0.075 g KNO_3, trace element solutions in 100 mL of distilled water, adjusted to pH 6.0.

Screening for avicelase and xylanase activities

Avicelase and xylanase activities were evaluated by inoculating in each case a plug (diameter 8 mm) of actinomycete S2 strain on either Avicel or beechwood xylan agar medium. Thereafter, incubation was carried out at temperature of 25–27 °C for 4 days. The enzymatic index (EI) was determined by Gram's iodine test (Kasana *et al*., 2008) and calculated as:

$$EI = \frac{\text{diameter of clearing zone} - \text{diameter of colony}}{\text{diameter of colony}}$$

Effect of Cu^{2+} ions and ascorbic acid on xylanase activity

Characteristics of xylanase activity as an indicator of LPMO were determined by examining the effect of Cu^{2+} ions and ascorbic acid on xylanase reactions using beechwood xylan as a substrate. Three plugs of actinomycete S2 strain culture were inoculated in 100 mL of 1% (w/v) beechwood xylan medium and incubated on a shaker (110 rpm) at temperature of 25–27 °C for 4 days. The crude xylanase extract was obtained by centrifuging the culture at 13,680 × *g* (4 °C) for 20 min. Xylanase activity was conducted by measuring the reducing sugar, which derived from the degradation of beechwood xylan.

Reducing sugar was determined by the DNS method as described by Miller (1959). Briefly, a 0.5 mL aliquot of crude xylanase extract was added to 0.5 mL of 1% (w/v) beechwood xylan in 100 mM Na-phosphate buffer, pH

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6.0, and incubated at room temperature for 15 min. The effect of Cu^{2+} ions was assayed by adding $CuSO_4$ solutions in final concentrations of 1 mM, 5 mM, and 10 mM, while the electron donor effect was tested by adding ascorbic acid to final concentrations of 0.5–2 mM. Both copper and ascorbic acid supplements were added to the enzymatic reactions prior to the incubation step. The reactions were stopped by the addition of 1.5 mL 3.5-dinitro-salicylic acid reagent to the mixture and boiling for 10 min. Optical density of the mixtures was recorded at 540 nm using a UV/Vis spectrophotometer and read against a standard xylose curve. One unit (U) of xylanase activity was defined as the amount of enzyme that released of 1 µmol of reducing sugar from beechwood xylan per minute (He *et al*., 2014).

Optimization of crude LPMO production based on reducing sugar analysis

Since the reducing sugar, xylose, is released through the synergistic interaction between LPMO and xylanase (one of the hydrolytic enzymes in the crude LPMO extract), LPMO activity could be determined by taking advantage of this reaction. Accordingly, the release of reducing sugar from Avicel (crystalline cellulose preparation that contains 2% xylan) (Frommhagen *et al*., 2015) can serve as an indicator for LPMO activity. Crude LPMO extract on Avicel medium was evaluated over several incubation durations to determine the optimum incubation period for crude LPMO extract production. Three plugs of fresh actinomycete S2 strain were cultivated in 100 mL of 1% (w/v) Avicel medium and incubated with shaking at 110 rpm for up to 10 days at temperature of 25–27 °C. Crude LPMO extract was prepared daily from the same culture as described above. Reducing sugar from the reaction of the crude LPMO extract with 1% (w/v) Avicel together with 1 mM ascorbic acid as an electron donor was determined as described above.

Determination of LPMO characteristic activities

In the production of the crude LPMO extract, 3 plugs of actinomycete S2 strain were inoculated onto 100 mL of 1% (w/v) Avicel media and incubated on a 110 rpm shaker at temperature of 25–27 °C for the optimal incubation time. The crude LPMO extract that was recovered as described above was used for determination of some characteristics on the enzyme preparation. The traits studied included xylanase and CMCase (carboxymethyl cellulase) activities, reducing sugar production and the effects of the electron donor (ascorbic acid), Cu^{2+} ions and metal binding. Carboxymethyl cellulose and beechwood xylan were used as the substrates for CMCase and xylanase activities, respectively. Xylanase and CMCase activities of actinomycete S2 strain was used for the initial step for screening the isolate character for LPMO activity. Of note, calibration of xylose and glucose standard curve was applied for determining xylanase and CMCase enzyme activities, respectively. One unit of CMCase activity was defined as the amount of enzyme that released of 1 µmol of glucose per-minute (Samira *et al*., 2011).

In assaying LPMO activity based on reducing-sugar production, *Bacillus thuringiensis* ATCC 33679 was used as a positive control owing to its three genes encoding LPMO (Zhang *et al*., 2015). Xylanase from *Pichia pastoris* KM71H was the negative control for LPMO activity. This is due to, previous report was succeeded to clone xylanase gene to this corresponding yeast (unpublished result) (Irawan Tan, private communication on August, 2018). Therefore, we could use this yeast to verify the effect of ascorbic acid in stimulating only LPMO activity, and not for the xylanase activity. Moreover, characterization of LPMO as copper-dependent enzyme was examined by adding 0.2 mM Cu^{2+} ions and/or 1 mM ascorbic acid to the LPMO enzyme reaction. In addition, metal binding activity was tested by supplementing 10 mM of EDTA to the enzyme reaction (He *et al*., 2014). The DNS method was applied in all reducing sugar production analysis and 1% (w/v) Avicel was used as the substrate.

Assay of LPMO activity based on H_2O_2 production

LPMO activity was followed up by testing H_2O_2 production using the Amplex Red fluorometric assay, with the crude enzyme preparation of *P. pastoris* KM71H and *Escherichia coli* serving as negative controls. The enzyme assays were started by mixing 20 µL of the crude LPMO extract/controls with 180 µL of assay solution containing 100 mM sodium phosphate buffer pH 6.0, 30 µM ascorbic acid, 50 µM Amplex Red (Sigma) and 7.14 $U.mL^{-1}$ horseradish peroxidase (Sigma) in a black 96-well plates (total assay volume of 200 µL). All the solutions were mixed and incubated at temperature of 25–27 °C for 15 min in the dark condition. Fluorescence was then measured based on resorufin production using excitation and emission wavelengths of 569 nm and 585 nm, respectively, of the fluorescence plate reader (BMG Lab Tech, USA). A linear relation between fluorescence and H_2O_2 concentrations in the range of 0.1–5 µM of H_2O_2 was constructed for standard calibration. One unit of LPMO activity was defined as the equivalent to the release of 1 µmol of H_2O_2 per-minute (Kittl *et al*., 2012).

Molecular identification of actinomycete S2 strain

The genomic DNA of the actinomycete S2 strain was extracted following a standard protocol for actinomycete genomic DNA preparations using the Genomic DNA Mini Kit (Geneaid). The 16S rRNA gene was amplified by polymerase chain reaction (PCR) (94 °C for 4 min, 30 cycles consisting of 94 °C for 45 sec, 55 °C for 60 sec, 72 °C for 70 sec followed by a terminal incubation at 72 °C for 7 min) using 63f (5′-CAG GCC TAA CAC ATG CAA GTC-3′) and 1387r (5′-GGG CGG WGT GTA CAA GGC-3′) primers (Marchesi *et al*. 1998). The 16S rRNA gene sequences of the strains were then searched for homology with sequences in the National Center Biotechnology Information (NCBI) public database using

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nucleotide blast (BLASTn) tool (http://www.ncbi.nlm.nih.gov/). Subsequently, the raw sequencing data were trimmed and assembled using ChromasPro program version 1.5. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 7 and the phylogenetic tree was constructed using the Neighbor-Joining method with a 1000× replication bootstrap.

RESULTS AND DISCUSSION

Actinomycete S2 strain exhibits avicelase and xylanase activities

In the initial analysis, we showed that actinomycete S2 strain displayed avicelase and xylanase enzyme properties with enzyme activity indices of 2.67 and 3.87, respectively (Figure 1). The results indicated that this actinomycete strain could utilize Avicel as a carbon source for its growth and LPMO activity was established by the ability of the enzyme to degrade Avicel (Agger *et al*., 2014; Frommhagen *et al*., 2015). In addition, the observed activity of xylanase, as one of the hydrolytic enzymes in lignocellulose biodegradation, could also be used as an indicator of LPMO activity assay based on the release of reducing sugars as a reaction product.

Effect of Cu^{2+} ions and ascorbic acid on xylanase activity

Xylanase activity of actinomycete S2 strain was further investigated using beechwood xylan as the substrate. We showed that actinomycete S2 strain xylanase activity of 0.549 ± 0.079 $U.mL^{-1}$ decreased by up to 73% (0.150 ± 0.024 $U.mL^{-1}$), 84% (0.089 ± 0.013 $U.mL^{-1}$) and 93% (0.0380 ± 0.12 $U.mL^{-1}$) following Cu^{2+} supplementation of 1 mM, 5 mM and 10 mM Cu^{2+}, respectively. On the other hand, xylanase activity increased up to nine fold, from 0.343 ± 0.048 $U.mL^{-1}$ to 3.315 ± 0.189 $U.mL^{-1}$ in the presence of 1.5 mM ascorbic acid (Figure 2). We surmise that the increase of xylanase activity of the actinomycete in the presence of ascorbic acid was due to its role as an electron donor for the LPMO reaction. Consistent with our findings, previous research has reported that the glycoside hydrolase family 61 (GH61) of *Thermoascus aurantiacus* (*Ta*GH61) increased the conversion of microcrystalline cellulose to oxidized cello-oligomers in C1 when gallic acid, another electron donor, was present in the reaction mixture. This particular enzyme is classified as polysaccharide monooxygenase (Phillips *et al*., 2011; Dimarogona *et al*., 2012). Additionally, an LPMO from *Neurospora crassa* (*Nc*LPMO09C) has been reported to carry out a similar oxidative reaction with xyloglucan. Structural similarity between (1→4)-β-D-xylan and (1→4)-β-D-glucan may indicate that LPMO is involved in xylan degradation (Agger *et al.,* 2014). On the contrary, the xylanase activity of actinomycete S2 strain declined after ascorbic acid supplementation was increased to 2 mM. This was presumably due to the higher concentration of ascorbic acid impacting not only LPMO activity directly, but also influencing its synergistic interaction with xylanase activity at that concentration.

Duration for optimal production of Actinomycete S2 strain crude LPMO

Microbes produce various enzymes in response to the presence of substrate in their growth media. The growth of actinomycete S2 strain on Avicel medium apparently stimulated the biosynthesis of crystalline-cellulose-degrading enzymes due to cellulose being the only source of carbon available in the medium for its growth. LPMO production was maximal after incubation for six days, with the release of 276.278 ± 14.404 $\mu g.mL^{-1}$ xylose as one of reducing sugar in Avicel substrate and in the presence of ascorbic acid (Figure 3). The LPMO activity was shown to increase three-fold by when ascorbic acid was present in the enzymatic reaction. While *Bacillus choshinensis* SP3, a mutant developed from the transformation of the *sg*LPMO10F gene of *Streptomyces griseus* could produce LPMO after 24 h of cultivation (Nakagawa *et al.*, 2015). The actinomycete S2 strain used in this study required more time for enzyme production.

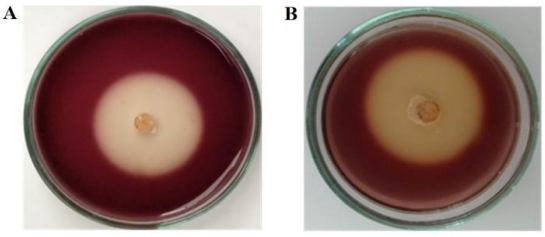

Figure 1: The Avicelase (A) and xylanase (B) activities of actinomycete S2 strain on Avicel and xylan media pH 6.0, respectively. Incubation at room temperature for four days was conducted prior to be observed. All figures are representative from triplicates experiments.

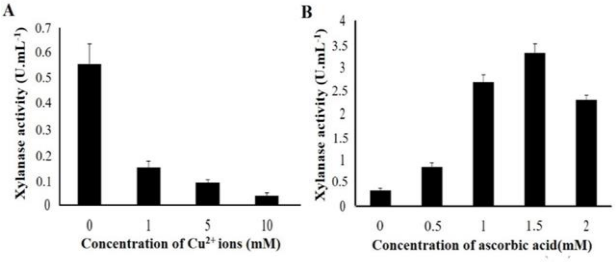

Figure 2: Effect of Cu^{2+} ions (A) and ascorbic acid (B) on xylanase activity of actinomycete S2 strain in beechwood xylan growth medium after cultured for 4 days at room temperature. Enzyme activity was determined using beechwood xylan as a substrate in Na-phosphate buffer, pH 6.0, and incubated at room temperature for 15 minutes. Results were represented from three independent experiments.

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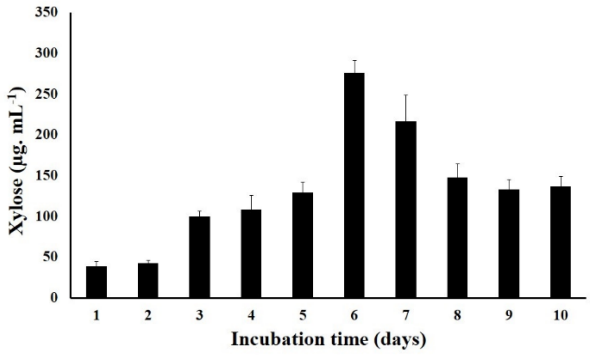

Figure 3: Effect of incubation time on LPMO production from actinomycete S2 strain. The LPMO activity was determined based on reducing sugar production. The LPMO activity assay was carried out in 1% (w/v) Avicel as for enzymatic substrate and in the presence of 1 mM ascorbic acid. Data are means and representative from triplicate experiments.

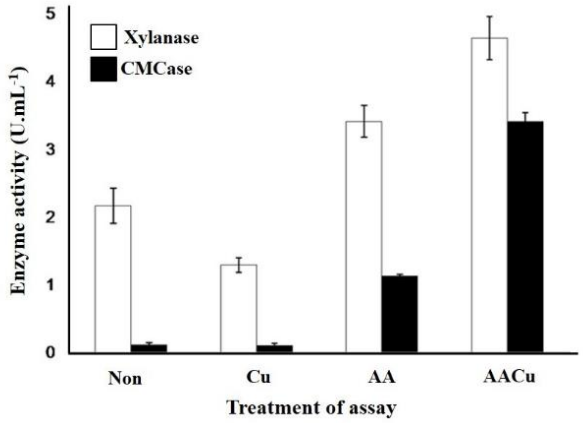

Figure 4: Comparison of crude extract xylanase and CMCase activity of actinomycete S2 strain on 1% (w/v) beechwood xylan and CMC as for enzymatic substrate, respectively, following the addition of Cu^{2+} ions and ascorbic acid. These crude enzymes were produces in 1% (w/v) Avicel growth medium. Non= without treatment; Cu= with Cu^{2+} ions; AA= with ascorbic acid; AACu= with ascorbic acid and Cu^{2+} ions. Data are means and representative from three independent experiments.

LPMO activity of actinomycete S2 strain determined by reducing-sugar production

The fact that xylanase activity increased in the presence of ascorbic acid provided a strong indicator that actinomycete S2 strain had LPMO activity. Frommhagen *et al*. (2015) had earlier reported that the release of glucose produced by enzymatic reaction of cellulase was increased in the presence of LPMO from *M. thermophila* (*Mt*LPMO9A). Interestingly, we found that xylanase and cellulase activities of actinomycete S2 strain produced on 1% (w/v) Avicel medium increased after the addition of ascorbic acid and the combination of ascorbic acid and Cu^{2+} ions in their substrate. With the addition to ascorbic acid, xylanase activity of actinomycete S2 strain was 3.41 ± 0.23 $U.mL^{-1}$ and that of CMCase was 1.17 ± 0.02 $U.mL^{-1}$. The combination of ascorbic acid and Cu^{2+} ions resulted in activities of 4.64 ± 0.32 $U.mL^{-1}$ and 3.41 ± 0.13 $U.mL^{-1}$ for xylanase and CMCase activities, respectively. However, the addition of Cu^{2+} ions alone decreased the activity of xylanase and CMCase to 1.29 ± 0.11 $U.mL^{-1}$ and 0.1 ± 0.02 $U.mL^{-1}$, respectively (Figure 4).

Additional assays using Avicel as enzymatic substrate were conducted to elucidate further the effect of copper and ascorbic acid on actinomycete S2 strain enzyme activity. Since Avicel contained 2% xylan, it would be possible to observe actinomycete S2 strain xylanase activity in Avicel substrate to examine the action of synergistic agents on the activity of the LPMO in crude extract enzyme (Frommhagen *et al*., 2015). In this investigation, crude extract enzymes from *B. thuringiensis* ATCC 33679 and *P. pastoris* KM71H were used as controls. Our results indicated that xylose was released from the activity of crude extract enzyme of *B. thuringiensis* ATTC 33679 and actinomycete S2 strain at a basal level of 175.01 ± 7.20 $\mu g.mL^{-1}$ and 255.03 ± 4.30 $\mu g.mL^{-1}$, respectively. Intriguingly, the xylose increased by 7-fold to 1150 ± 8.82 $\mu g.mL^{-1}$ for *B. thuringiensis* ATCC 33679 and 3-fold to 782.5 ± 26.30 $\mu g.mL^{-1}$ for actinomycete S2 strain when ascorbic acid was present in the Avicel reaction mixture (Figure 5). In contrast, xylose released from the xylanase activity of *P. pastoris* KM71H decreased up to 40%, from 1005.56 ± 78.76 $\mu g.mL^{-1}$ to 605.57 ± 25.46 $\mu g.mL^{-1}$ when ascorbic acid was added in the Avicel. Moreover, a combination of 1 mM ascorbic acid and 0.2 mM Cu^{2+} ions increased xylose produced by *B. thuringiensis* ATCC 33679 by 8-fold to 1435 ± 68.61 $\mu g.mL^{-1}$ and 5-fold to 1290 ± 75.62 $\mu g.mL^{-1}$ for actinomycete S2 strain, respectively. However, the same treatment decreased the xylose from xylanase activity of *P. pastoris* KM71H by 59% (416.67 ± 33.33 $\mu g.mL^{-1}$) (Figure 5). It is noteworthy that synergistic reactions play an important role in the biodegradation of lignocellulose. In addition to the hydrolytic enzymes that degrade cellulose or hemicellulose, LPMO that has the ability to oxidize crystalline cellulose especially if an electron donor is present in an enzymatic reaction (Andlar *et al*., 2018). LPMO activity is specific not only to crystalline cellulose, but also to amorphous cellulose, hemicellulose, chitin and other polysaccharides (Agger *et al*., 2014). In general, biomass degrading bacteria have only one or two LPMO-encoding genes, but *Streptomyces coelicolor* is reported as having seven genes encoding LPMO while *S. griseus* has six LPMO genes (Nakagawa *et al*., 2015). The finding that ascorbic acid as an electron donor increased the production of reducing sugars and hydrolytic enzyme activity (CMCase and xylanase) were good indicators that actinomycete S2 strain produced LPMO (Figure 4). We also showed that the enzyme activity and reducing sugar product from actinomycete S2 strain was increased in presence of ascorbic acid and Cu^{2+} ions in combination. This suggests that LPMO was copper-dependent needed

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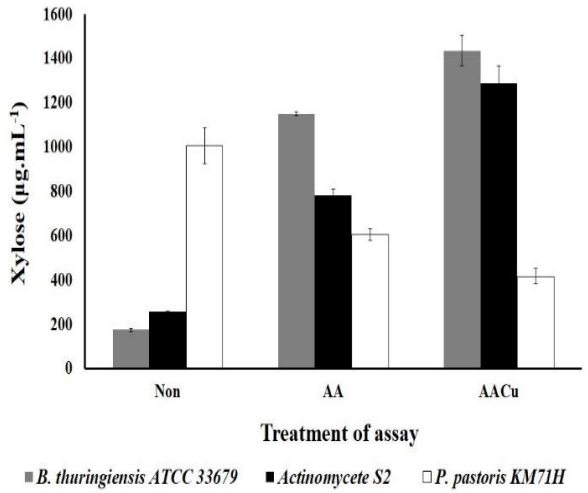

Figure 5: Effect of ascorbic acid and Cu^{2+} ions on LPMO crude extract of *B. thuringiensis* ATCC 33679, actinomycete S2 strain and *P. pastoris* KM71H based on reducing sugar production on Avicel 1% following the addition of Cu^{2+} ions and ascorbic acid. Non = normal assay without Cu^{2+} ions and ascorbic acid; AA = with ascorbic acid; AACu = Cu^{2+} ions and ascorbic acid addition. Results are means and representative from three independent experiments.

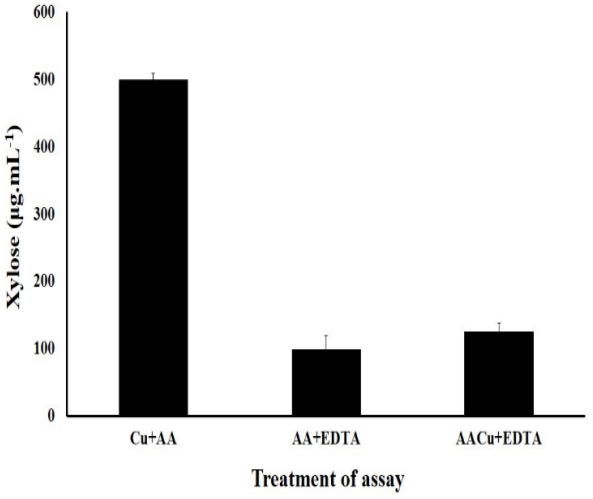

Figure 6: Metal-binding assay of actinomycete S2 strain LPMO activity on 1% Avicel based on reducing sugar production. Cu+AA= addition of Cu^{2+} ions and ascorbic acid; EDTA= addition of ascorbic acid and EDTA; CuAA+EDTA= addition of ascorbic acid, EDTA and Cu^{2+} ions. Data are means and representative from three independent experiments.

a confirmatory metal-binding test. The decrease in xylanase activity with the addition of Cu^{2+} ions, in the absence of ascorbic acid as shown in Figure 1, might also point to copper as one of the inhibitors for xylanase activity of actinomycete S2 strain.

The dependence of LPMO activity on copper can be demonstrated by the addition of EDTA (a cation-chelating agent) in the enzymatic reaction (Figure 6). The results showed that 10 mM EDTA decreased xylose production of LPMO of actinomycete S2 strain by 81%, from 500.28 ± 8.53 µg.mL^{-1} to 98.61 ± 19.73 µg.mL^{-1}. However, when Cu^{2+} ions were added to the enzymatic reaction, there was a recovery in the production of xylose by 27%, with an increase to 125.56 ± 13.07 µg.mL^{-1} (Figure 6). Our results are, therefore, consistent with previous findings that the glycoside hydrolase activity of *Phanerochaete chrysosporium* (*Pc*GH61D), produced by oxidizing cello-oligosaccharides following the addition of ascorbic acid, was inhibited by the addition of EDTA, but a recovery ensued following the addition of copper and manganese (Westereng *et al*., 2011). Indeed, Nakagawa *et al*. (2015) and Kracher *et al*. (2016) note that the active site of LPMO contains two histidines which bind to copper atom. Thus, Cu^{2+} ions play a critical role in the oxidation process, where the oxygen activation reaction at this active site requires an electron donor.

Actinomycete S2 strain LPMO activity based on H_2O_2 production

In the absence of cellulose as substrate, LPMO activity can also be detected quantitatively based on its by-product, H_2O_2 (Kittl *et al*., 2012). Here, we further investigated the LPMO actinomycete S2 strain characteristics in terms of H_2O_2 production (Table 1). Using the Amplex Red Fluorimetric assay to quantify H_2O_2 production, the crude enzyme from *B. thuringiensis* ATCC 33679 had LPMO activity of 0.029 ± 0.004 U.mL^{-1}, while LPMO activity of actinomycete S2 strain was 0.019 ± 0.001 U.mL^{-1}. LPMO activity from the negative controls *P. pastoris* KM71H and *E. coli* was not detected. However, LPMO activity of actinomycete S2 strain was comparatively lower than that reported for *Neurospora crassa* (4.90 U.mL^{-1}) (Kittl *et al*., 2012). Nonetheless, our results confirmed LPMO enzyme in our sample through H_2O_2 production in addition to reducing-sugar production.

Identification of actinomycete S2 strain as *Streptomyces* sp.

Gene amplification of actinomycete S2 strain by PCR with 63f and 1387r primers produced DNA fragments of around 1300 bp (Gene Bank deposit number MK050007.1). BLASTn analysis of the 16S rRNA gene sequence of actinomycete S2 strain showed 99% similarity to the 16S rRNA gene sequence of *Streptomyces avermitilis* EAAG80 strain (Table 2). Actinomycete S2 strain also has genetic proximity to *S. yokosukanensis* strain EAAG75, *S. cellostaticus* strain CSSP188, and *S. griseochromogenes* strain NBRC 13413 (Figure 7). It is noteworthy that *S. griseochromogenes* ATCC 14511 also possesses an LPMO-encoding gene (Lijuan *et al*., 2017).

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Table 1: The LPMO activity of actinomycete S2 strain based on H_2O_2 production. LPMO activity from *B. thuringiensis* ATCC 33679 utilized as positive control, while both *P. pastoris* KM71H and *E. coli* were used as negative controls. One unit LPMO activity is defined as the equivalent value of the release of 1 µmol of H_2O_2 per-minute. Data are means and representative from three independent experiments.

Isolates	H_2O_2 production (µmol. L^{-1}.min^{-1})			LPMO activity ($U.mL^{-1}$)**
	With ascorbic acid	No ascorbic acid	Final H_2O_2*	
B. thuringiensis ATCC 33679	0.0135 ± 0.0008	0.0077	0.0058 ± 0.0008	0.029 ± 0.004
Actinomycete S2 strain	0.0121 ± 0.0002	0.0081	0.0039 ± 0.0002	0.019 ± 0.001
P. pastoris KM71H	0.0033 ± 0.00	0.0033	0.000	0.000
E. coli	0.0007 ± 0.00	0.0007	0.000	0.000

*Final H_2O_2 obtained by subtracting mol of H_2O_2 production with ascorbic acid and no ascorbic acid treatment.
**LPMO activity ($U.mL^{-1}$) obtained by converting final mol of H_2O_2 production of each sample from reaction of 200 µL (total assay volume) to mL volume (for U. mL^{-1}), in detail each of H_2O_2 final production is multiplied with 1000 (converting to mL), then dividing with 200 (µL); Ascorbic acid used for an electron donor; *B. thuringiensis* ATCC 33679 used for positive control of LPMO activity (contained LPMO genes); *P. pastoris* KM71H used for comparison treatment which have xylanase gene; *E. coli* used for comparison treatment which have no direct relationship with LPMO/xylanase. Each assay was conducted in 200 µL volume.

Table 2: The alignment gene of 16S rRNA sequence between actinomycete S2 strain and other species based on sequence data in NCBI (BLASTn).

Relative species	Query cover (%)	E value	Identity (%)	Accession number
Streptomyces avermitilis strain EAAG80	100	0.0	99	KF562159.1
Streptomyces yokosukanensis strain EAAG75	100	0.0	99	KF032543.1
Streptomyces cellostaticus strain CSSP188	100	0.0	99	NR_043339.1
Streptomyces griseochromogenes strain NBRC 13413	100	0.0	99	NR_112396.1

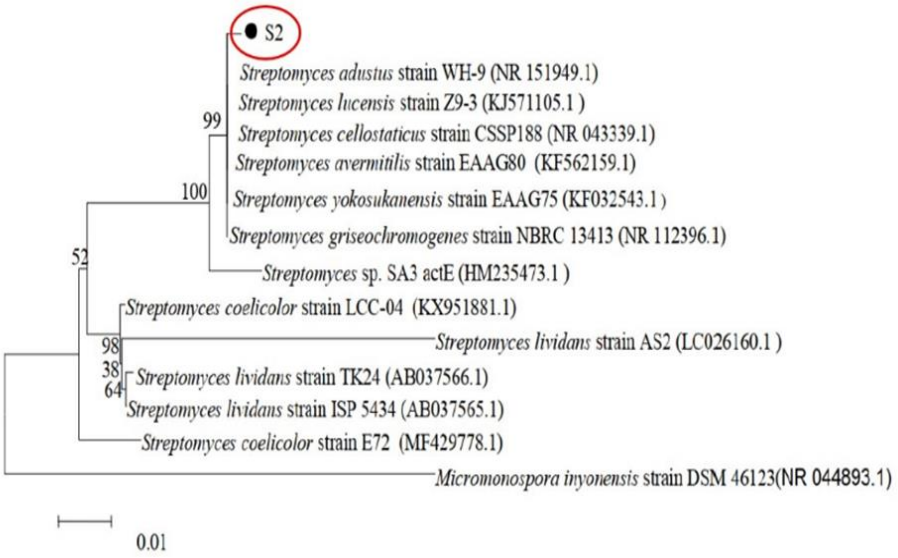

Figure 7: Phylogenetic tree (dendrogram) of actinomycete S2 strain 16S rRNA gene sequences using the neighbor-joining method. Bootstrap test= 1000 replicates. The evolutionary distances were computed using the Tamura model. Analysis was conducted using Mega 7 software.

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CONCLUSION

Actinomycete S2 strain is capable of producing LPMO enzyme which can be detected based on the release of reducing-sugar and the formation of hydrogen peroxide. This reaction being enhanced by the addition of ascorbic acid as an electron donor and Cu^{2+} ions to the enzymatic reaction. On the basis of the 16S rRNA gene analysis, actinomycete S2 strain bears a close relationship to *S. avermitilis* strain EAAG80, with about 99% similarity. As far as we are able to ascertain, this is the first elucidation of LPMO activity from an indigenous Indonesian actinomycete.

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