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Pretreated Sugarcane Bagasse Result in more Efficient Degradation by *Streptomyces* sp S2

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Streptomyces genera plays an important role in lignocellulose degradation. Many research found that *Streptomyces* have cellulolytic and ligninolytic enzymes that are sufficient to degrade lignocellulosic materials. However, a minimum lignocellulosic material condition that can efficiently be degraded by *Streptomyces* sp. has not been fully understood. In this research, three pretreatment conditions (physical, alkaline-hydrothermal, and hydrogen-peroxide chemical treatments) of sugarcane bagasse were used as lignocellulosic material to be further degraded by *Streptomyces* sp. S2. Lignocellulose component measurement concluded that raw (physically treated only) bagasse wasn't efficiently degraded by *Streptomyces* sp S2. Hydrogen-peroxide was effective in reducing both syringic and guaiacyl lignin. Meanwhile, alkaline-hydrothermal pretreatment was very effective in lowering syringic lignin. This study suggests that hydrogen-peroxide pretreatment can be used in any type of lignocellulosic material, which can be further degraded by *Streptomyces* sp. S2. On the other hand, Alkaline-hydrothermal pretreatment is best suited to degrade lignocellulosic material with a high percentage of syringic lignin.

Key words: Alkaline-hydrothermal treatment, Hydrogen peroxide treatment, lignocellulose, *Streptomyces* sp. S2, sugarcane bagasse

Genus *Streptomyces* berperan penting dalam degradasi lignoselulosa. Penelitian terdahulu telah membuktikan bahwa *Streptomyces* memiliki enzim selulolitik dan lignolitik yang dapat mendegradasi bahan lignoselulosa. Namun hingga saat ini belum diketahui kondisi awal bahan lignoselulosa yang sesuai agar dapat didegradasi secara efisien oleh *Streptomyces*. Pada penelitian ini, bagas tebu sebagai sumber lignoselulosa mendapatkan tiga macam perlakuan pendahuluan (perlakuan fisik, perlakuan alkali-hidrotermal dan perlakuan kimiawi hidrogen peroksida), dan kemudian diikuti dengan degradasi oleh *Streptomyces* sp. S2. Berdasarkan hasil analisis komponen lignoselulosa memperlihatkan bahwa perlakuan fisik melalui pengecilan ukuran belum dapat mendegradasi secara efisien. Perlakuan hidrogen-peroksida secara efektif dapat menurunkan komponen syringil dan guaiacyl lignin, sementara perlakuan alkalin-hidrotermal hanya efektif menurunkan komponen syringic lignin. Kajian ini memperlihatkan pengaruh perlakuan hidrogen peroksida dapat digunakan untuk berbagai bahan lignoselulosa, kemudian dilanjutkan dengan degradasi oleh *Streptomyces* sp. S2; sedangkan perlakuan alkali-hidrotermal dapat mendegradasi bahan lignoselulosa dengan persentase syringic lignin yang tinggi.

Kata kunci: bagas tebu, degradasi lignoselulosa, perlakuan alkali-hidrotermal, perlakuan hidrogen peroksida, *Streptomyces* sp. S2

Cellulose fiber with hemicellulose fiber and lignin together form lignocellulose materials. In recent years, bioethanol, biogas, bioplastics, and simple sugar were derived from lignocellulose materials (Azeredo *et al*.2015; Machado and Ferraz 2017; Fan *et al.* 2018; Sari *et al*.2021). Indonesia has significant potential to use lignocellulose material since it is an agricultural country. In 2017, about 150 million tonnes of dried rice grain, 30.000 thousands tonnes of corn and 2000 thousands of sugarcane were produced in Indonesia (Kementerian Pertanian Republik Indonesia 2018). Bioethanol and bioplastics can be produced from sugarcane bagasse, since sugarcane bagasse has high performance index and low environmental effect (Sari *et al.* 2021). However, 17.52%-23.37% of lignin in sugarcane bagasse makes utilization of its cellulose to simple sugar hampered (Maryana *et al.*2014; Pin *et*

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*al.*2019). High crystallinity and high polymeryzation degree of sugarcane bagasse cellulose also builds sugarcane bagasse strength as lignocellulosic material. Thus, the accessible cellulose area becomes small (Lee *et al.* 2014).

Pretreatment and/or bleaching can be used to reduce lignin content. The bleaching process has been used in the paper industry, reduce lignin content and brighten lignocellulose color. Usually the bleaching process use chemicals such as H_2O_2, $NaClO_2$, or ClO_2, which can contain chlorine or not. Pretreatment focuses are to reducing lignin content and reducing the size of lignocellulose particles. Some pretreatments types are physical (extrusion, ultrasonic radiation, milling), physicochemical (supercritical CO_2, ammonia fiber explosion). Chemical pretreatment utilizes alkaline or acid solution in high concentration or dilute, ionic liquid, deep-eutectic-solvent (DES) or organosolv. Biological pretreatment uses whole organisms (bacteria or fungi) or their enzymes to degrade lignin (Baruah *et al.*2018; Septevani *et al.*2018; Singh *et al.*2019).

Streptomyces sp. is Gram-positive bacteria that live in soil and extreme environments such as volcanoes, deep sea and extremely cold places like the Arctic. Most *Streptomyces* sp. produce secondary metabolites, such as anti-cancer and antibacterial, also can degrade substrate in dead plants (Chater 2016; Sivalingam *et al*.2019). *Streptomyces,* alongside *Xanthomonas, Pseudomonas* and *Flavobacteria,* are major lignocellulose degraders on the first three days of degradation (Ma *et al*. 2020). *Streptomyces* ability to degrade plant biomass is influenced by the high number of binding sites called CebR. Enzymes associated with plant biomass deconstruction such as endoglucanase, glycoside hydrolase, β-glucosidase, cellobiohydrolase, xylanase, mannanase, chitinase and LPMO (lytic polysaccharide monooxygenase) is determined by the amount of the CebR sites (Book *et al.* 2016). Ligninolytic enzymes such as lignin-peroxidase (LiP), low laccase (Lac), dye decoloring enzyme (DyP), and aryl alcohol oxidase (AAO) activities can be found in ligninolytic *Streptomyces*, but without manganese-peroxidase (MnP) enzyme activities (Riyadi *et al.* 2020).

Streptomyces sp. is potentially used to degrade lignocellulose material, since it has cellulolytic and ligninolytic enzyme activities. However, *Streptomyces* sp degradation ability on different lignocellulose conditions hasn't been fully understood. Biological degradation was done using *Streptomyces* sp S2 that isolated from oil palm plantation in Jambi, Indonesia. *Streptomyces* sp S2 degradation ability had been tested on three sugarcane conditions (control (milled only), alkaline-hydrothermal, hydrogen-peroxide). Alkaline-hydrothermal and hydrogen-peroxide pretreatment are widely used to reduce lignin content. Both pretreatment can make bagasse easier to degrade by *Streptomyces* sp S2, compared to milled treated-only bagasse as control. Each sugarcane bagasse condition was evaluated its crude fiber content, lignocellulose component, crystallinity using X-ray diffraction before and after degraded by *Streptomyces* sp S2. The bagasse chemical contents were analyzed using Fourier Transform Infrared, and lastly its 3D structure was scanned using Scanning Electron Microscope (SEM). Amount of reducing sugar, total sugar and polymerization degree were also measured before and after enzymatic degradation using *Streptomyces* sp S2.

MATERIALS AND METHODS

Sugarcane Bagasse Preparation. Total of 40 kg wet weight of sugarcane bagasse from sugarcane juice seller in Bogor, was cut, washed until clean, then sun-dried for 3 days, or accelerated using oven until its water content was lower than 9%. All dried bagasse was then size reduced using Wiley Mill, filtered through 40 mesh sieve. Bagasse that had passed through the sieve was used for further treatment.

Alkaline-Hydrothermal Pretreatment. Each 100 g prepared bagasse was put into 2L Erlenmeyer and treated with 1.5 L 1M NaOH (solid to liquid ratio 1:1.5) . Erlenmeyer was closed using rubber bands and plastic, before being heated in an autoclave at 121°C for 30 minutes (Lemoes *et al.* 2018 modified temperature used). The alkaline-autoclaved pretreated bagasse was washed using aquadest until the neutralized pH solution was same or near as the aquadest used (pH 5.5). Washed sugarcane bagasse was filtered using filter paper and then dried using 60-80°C oven.

Hydrogen Peroxide Pretreatment. Every 100 g sieved bagasse (using 40 mesh sieve) was treated with 1% (w/v) trisodium citrate dihydrate, 6% (w/v) H_2O_2, 1% NaOH (w/v) and 92% (w/v) aquadest with total solution of 1L in 2L Erlenmeyer. Erlenmeyer was closed using holed-plastics for gas release that formed during the pretreatment and tightened using rubber bands. Peroxide treatment was done in a waterbath at 60°C for 90 minutes (Yan *et al*. 2019). Peroxide pretreated bagasse was further washed using aquadest until the solution pH was near or same as the aquadest used (pH 5.5). Bagasse that had been washed then

filtered before dried on a 60-80°C oven.

Application using *Streptomyces* sp. S2

Culture Preparation. *Streptomyces* sp S2 strain is indigenous to Indonesia and found to have Lytic Polyssacharide Monooxygenase (LPMO) activities that help to open cellulose bonds (Utarti *et al.* 2020). *Streptomyces* sp.S2 was inoculated into ISP 4 agar medium, incubated for 5 days in room temperature.

Degradation using *Streptomyces* sp. S2. Prepared culture of *Streptomyces* sp. S2 then moved using a 10 mm diameter corkborer into 200 ml ISP 4 liquid medium (pH=6.0) then incubated for 14 days in 100 rpm and 27°C shaker. After the incubation, the liquid medium containing *Streptomyces* sp. S2 was poured into 500 ml Erlenmeyer containing 15 g of previously sterilized (autoclaved 125°C, 40 minutes) sugarcane bagasse from previous pretreatment (control, alkaline-hydrothermal treated, peroxide treated). Degradation of treated bagasse using *Streptomyces* was taken 4 days. On the 4th days of bagasse degradation, bagasse was filtered through filter paper before being put into the freezer, to stop enzymatic degradation. Each pretreatment (control, alkaline-hydrothermal, hydrogen-peroxide) was treated duplo for *Streptomyces* sp. S2 pretreatment.

Enzymatic Degradation using *Streptomyces* sp S2 Crude Enzyme. One 10 mm corkborer of prepared *Streptomyces* sp. S2 was moved into 30 ml ISP-4 liquid medium, for enzyme production. Optimum amount of *Streptomyces* sp. S2 crude enzyme was added to control, alkaline pretreated and peroxide pretreated bagasse in different ratios (1:2; 1:3; 1:4). Enzymatic degradation was done at 120 rpm and 27°C shaker, incubated for four days. Measurement of total sugar, reducing sugar and observation of fiber was done after four days of incubation. Measurement of total sugar after enzyme incubation was done using Miller (1959) DNS method, meanwhile, total sugar was done using Dubois *et al.* (1956) phenolic sulfate method. Observation of bagasse fiber was done by observing fiber under the microscope. Degree of polymerization was based on total sugar divided by reducing sugar.

Fiber Characterization

Crude Fiber Measurement. Crude fiber was measured using AOAC method (1998). Crude fiber content calculated based on the formula:

$$\text{Crude fiber: } \frac{lignocellulose\ weight}{sample\ weight}\ x\ 100\% \qquad (1)$$

Neutral Detergent Fiber. Both Neutral Detergent Fiber and Acid Detergent Fiber were measured using AOAC (1998) method. Each 1 gram sample (a) and 100 ml neutral detergent solution was added into a 600 ml beaker. The sample and solution were heated until they boiled. After 60 minutes of boiling time, the sample was extracted and filtered through a fritted disc (b). Residues were washed using acetone and water, then further dried using an oven at 105°C until stable weight (c), then calculated using the formula:

$$NDF(\%) = \frac{c\text{-}b}{a}\ x\ 100\% \qquad (2)$$

Acid Detergent Fiber and Hemicellulose. Each gram of sample (a2) and 100 ml acid detergent solution was added into a 600 ml beaker, then heated until boiled. The sample was then extracted after 60 minutes of boiling time, filtered through a fritted disc (b2). Residues were washed using water and acetone, then further dried using 105°C oven until stable weight (c2). Each acid detergent fiber and hemicellulose calculated using the formula:

$$ADF(\%) = \frac{c2\text{-}b2}{a2}\ x\ 100\% \qquad (3)$$

$$Hemicellulose(\%) = NDF\ (\%) - ADF(\%) \qquad (4)$$

Cellulose Content Measurement. Sample from acid detergent fiber measurement (c2) was soaked in 72% H_2SO_4 for 3 hours. Residues that had been formed then was washed using hot water and acetone. Remaining sample was dried using a 105°C oven until the stable weight was achieved (d). Cellulose content was calculated using the formula:

$$Cellulose(\%) = \frac{c2\text{-}b}{a2}\ x\ 100\% \qquad (5)$$

Lignin Content. Sample from cellulose content measurement (d) was dried in a furnace at 600°C, until the stable weight was achieved (e). Lignin content was calculated using the formula:

$$Lignin(\%) = \frac{d\text{-}e}{a2}\ x\ 100\% \qquad (6)$$

Crystallinity Index Assay. Crystallinity index assay was done using XRD Shimadzu Maxima 7000 (Japan) in the Research Center of Biomaterials, National Research and Innovation Agency (BRIN), Cibinong, West Java, Indonesia. The sample was analyzed between 10° to 80° on continous scanning at 2° speed per minute, with Cu radiation (1. 506 Å). Background noise was separated from the selected XRD pattern, and then the X-ray pattern was corrected

and normalized using a computer. Crystallinity index was calculated using the formula: $(I_{cr} / (I_{cr} + I_a)) \times 100\%$, (7) where I_{cr} is crystalline area meanwhile I_a is amorph area.

Functional Group Analysis. Fourier-transform analysis was done using Spectrum Two, Perkin Elmer (USA) on absorbance between 400–4000 cm^{-1} and spectrum area between 4 cm^{-1} and 16 scans. Wave intensity was based on the percent of transmittance, which was calculated and observed later.

Morphological Characteristic. Each pretreatment sample was weighed around one gram (without repetition) and dehydrated using gradual ethanol (ethanol concentration: 50%, 70%, 80%, 90% and last 100%) before being coated with gold. Each gold-coated bagasse sample was observed on 200, 500 and 1000 times magnification using electron microscope SEM JEOL JSM-IT200 (Japan) at 3 kilo-Volt.

Analytical Statistics. Crude fiber and lignocellulose were duplo repeated, data were expressed as mean ± standard deviation. Data were further processed using ANOVA (analysis of variance) along with a t-test assuming equal variances with α=0.05 and α= 0.01, respectively. The amount of reducing sugar, total sugar and polymerization degree was duplo repeated, expressed as mean ± standard deviation and processed using ANOVA.

RESULTS

Lignocellulosic Component of Sugarcane Bagasse. Around 31.90% total dry weight of raw bagasse was crude fiber. Crude fiber of control bagasse was ineffectively degraded by *Streptomyces* sp S2, concluded from not statistically significant crude fiber between control and control-*Streptomyces*. Crude fiber of alkaline and peroxide pretreated bagasse was increased by 19.96% and 10.95%, respectively. NaOH-*Streptomyces* bagasse crude fiber was increased by 5%, which was not statistically significant to alkaline-hydrothermal pretreated bagasse. *Streptomyces* sp S2 can significantly decrease hydrogen-peroxide pretreated bagasse crude fiber by 4.6% (Table 1).

Raw sugarcane bagasse used in this research contains 46.65% cellulose, 31.83% and 9.90% lignin. Control bagasse cellulose content was increased by 2.51% after *Streptomyces* degradation; meanwhile, hemicellulose and lignin were slightly increased by 0.45% and 0.88%, respectively. Both alkaline-hydrothermal and hydrogen peroxide pretreatment reduces hemicellulose to 12.46% and 21.38%, respectively. On the other hand, the cellulose content of alkaline-hydrothermal and hydrogen peroxide treated bagasse was increased by 31.56% and 4.99%, respectively. However, lignin content was increased significantly by 6.34% after peroxide pretreatment, while lignin content decreased by 4.08% after alkaline pretreatment.

Streptomyces degradation to alkaline pretreated bagasse reduces cellulose significantly by 4.86% from 78.21% to 73.35%. On the other hand, *Streptomyces* degradation only slightly increases both hemicellulose and lignin content of alkaline pretreated bagasse. Cellulose content was increased by 1.52% to 53.16% and hemicellulose content was increased significantly by 3.87% to 25.25%, when peroxide bagasse was further degraded using *Streptomyces* sp S2. *Streptomyces* degradation reduces the lignin of peroxide bagasse significantly by 6.48% to 9.76% compared to peroxide treatment only, meanwhile not significant compared to control bagasse (Table 1).

Crystallinity index analysis. Raw bagasse cellulose consists of 54% cellulose distributed unevenly (amorph), while 45.34% was distributed in a well-ordered crystalline area. Alkaline-hydrothermal pretreatment caused a wider amorph area and loosened cellulose bonds, reducing crystallinity to 35.44%. Crystalline area after peroxide pretreatment was increased by 5%, which can also mean cellulose purity had increased.

Streptomyces sp S2 enzyme was more efficiently digest cellulose crystalline area in peroxide bagasse. This was indicated from higher crystalline area reduction (11% crystalline area reduction) in peroxide bagasse compared to only 9% crystalline area reduction in control bagasse, after both bagasse was degraded by *Streptomyces* . The crystallinity of alkaline pretreated bagasse remained stable after being degraded by *Streptomyces,* only slightly increased by 1.51% (Fig 1).

Functional Group Analysis. Compared to raw bagasse, both alkaline-hydrothermal and hydrogen-peroxide pretreatment can weaken bonds between 3500-3000 cm^{-1}. *Streptomyces* degradation increase peak number 3300 cm^{-1} of control and alkaline pretreated bagasse. On the other, *Streptomyces* decrease the intensity of peak number 3300 of peroxide pretreated bagasse. Band on 1726 cm^{-1} was found on raw bagasse; this bond was weakened after all treatment.

Raw bagasse has bonds between 1300-1200 cm^{-1},

with a higher 1300 bond than the 1200 cm^{-1}. The peak on 1300 cm-1 was reduced after the alkaline-hydrothermal treatment, but the peak on 1200 cm^{-1} wasn't. On the other hand, peroxide pretreatment had reduced both 1300-1200 cm^{-1} bonds. Although the drop on 1300 cm^{-1} was not as high as after alkaline pretreatment. *Streptomyces* degradation of peroxide bagasse can further weaken 1300 cm^{-1} and 1200 cm^{-1} (Fig 2). Both alkaline and peroxide treatment weaken bands between 1100-900 cm^{-1} compared to control and control-*Streptomyces* bagasse. *Streptomyces* degradation had increased the peak on 1160 cm^{-1} of previously alkaline pretreated bagasse; meanwhile, the same peak number of peroxide pretreated bagasse was decreased. A slight decrease of peak number 832 cm^{-1} was only found in peroxide-*Streptomyces* bagasse (Fig 2).

Morphological Characteristic. Raw sugarcane bagasse has been shown to have neatly and regularly bundled fiber, without difference in depth (Fig 3A). Alkaline-hydrothermal pretreatment creates multilayer folding (consists of several bundles of fiber) and big holes on the outer layer of bagasse (Fig 3B). Shallow holes were scattered around the outer layer of peroxide pretreated bagasse. Peroxide bagasse also has multilayer bundles with rough edges and differences in depth (Fig 3C). Control-*Streptomyces* bagasse shown to have some folding and deep holes from *Streptomyces* activities were scattered around its outer layer (red arrows, Fig 3D) . *Streptomyces* degradation to alkaline pretreated bagasse seems to remove lignin from the outer layer, leaving a smooth layer of bagasse and deep holes scattered around its surface (red arrows, Fig 3E). *Streptomyces* degradation creates deep holes on peroxide pretreated bagasse (red arrows, Fig 3F); most of the bagasse outer layer was also completely removed, exposing another layer of cellulose and hemicellulose.

Enzymatic Degradation using *Streptomyces* sp S2. Control sugarcane bagasse had the highest drop in polymerization degree (from 37 to 11) alongside the highest drop in total sugar, resulting in the highest reducing sugar amount on the highest enzyme concentration ratio (1:4). The significant drop in polymerization degree and total sugar, with a significant increase in reducing sugar of control, only happened in 1:4 enzyme ratio. Meanwhile enzyme ratio 1:3 wasn't change anything of control bagasse compared to the lowest (1:2) enzyme ratio. Alkaline pretreatment bagasse had the lowest polymerization degree at the lowest enzyme ratio (24 at 1:2 ratio), had a significant drop (to 13) as enzyme concentration increased to 1:4. The significant drop in polymerization degree of alkaline bagasse only happened on the highest enzyme concentration (1:4) compared to the lowest enzyme concentration to previous alkaline bagasse and control (1:2). Meanwhile drop in polymerization degree of alkaline bagasse (1:4 enzyme ratio) wasn't significant compared to when using 1:3 enzyme ratio. The lowest polymerization degree and most significant drop were achieved in peroxide pretreatment bagasse (8) on 1:4 bagasse to enzyme concentration ratio, decreased by 22 points from 30 on 1:2 enzyme concentration and 3 points from 1:3 enzyme concentration (Table 2).

The Highest hydrolysis efficiency was achieved by peroxide pretreatment bagasse on 1:4 enzyme concentration (highest percentage of reducing sugar from its total sugar amount), compared to raw bagasse and alkaline pretreated bagasse. Alkaline pretreated bagasse had the lowest hydrolysis efficiency (lowest reducing sugar produced from its total sugar amount); the significant drop in total sugar of alkaline bagasse only resulted in a slight increase of its total sugar.

DISCUSSION

Cellulose, hemicelulose, lignin and some amount of β-glucan, dextrin, pectin, mucilages, inulin, also oligosaccharides/ oligofructose are form crude fiber (Slavin *et al.* 2009). Alkaline pretreated bagasse increased cellulose content and reduced lignin was similar to Srivastava *et al.* (2017). Meanwhile, peroxide pretreated bagasse lignin content was increased, which was inconsistent compared to Yan *et al.* (2019) findings. Increased lignin content might be because sugarcane bagasse has high (around 130 kJ/mol) lignin enthalpy, which makes sugarcane has high lignin thermal stability. Higher thermal stability makes hydrogen-peroxide harder to extract bagasse lignin compared to other lignocellulose with lower thermal stability, within the same temperature (Chen *et al.* 2016; Watkins *et al.* 2015). Another possible explanation is lignin was moved to the surface of bagasse because of the acid nature of peroxide pretreatment. Lignin on the bagasse surface was easier to be accessed by bacteria, resulting in significant lignin content drop in peroxide bagasse when further treated using *Streptomyces* (Table 1). A wider specific surface area accessible to bacteria, caused by increased crystallinity, can be further digested by bacteria. These findings were consistent and similar to Xu *et al.* (2017)

Table 1 Crude fiber and Lignocellulose Component of Sugarcane Bagasse

Treatment	Crude Fiber (%)	Hemicellulose (%)	Cellulose (%)	Lignin (%)
Control	31.90 ± 0.50	31.83 ± 0.74	46.65 ± 0.66	9.90 ± 0.55
Alkaline-hydrotermal (NaOH)	51.86 ± 1.09^{b}	12.46 ± 1.09^{b}	78.21 ± 0.42^{b}	5.82 ± 1.00^{a}
Hydrogen-Peroxide (H_2O_2)	42.85 ± 0.93^{b}	21.38 ± 0.34^{b}	51.64 ± 0.59^{a}	16.24 ± 0.08^{b}
Control-*Streptomyces*	31.25 ± 1.03	32.28 ± 0.25	44.14 ± 0.91	10.78 ± 0.45
NaOH-*Streptomyces*	55.15 ± 0.67^{b}	13.20 ± 0.21^{b}	73.35 ± 0.21^{bb}	6.54 ± 0.31^{a}
H_2O_2-*Streptomyces*	38.25 ± 0.40^{ba}	25.25 ± 0.97^{ba}	53.16 ± 0.08^{b}	9.76 ± 0.18^{ns-b}

Hemicellulose percentage was based on neutral detergent fiber (NDF) substracted by acid detergent fiber (ADF); data were not shown.

$^{(a,b)}$The first superscript letter show statistical significance compared to control only,

$^{(a,b)}$ The second superscript shows statistical significance from its previous treatment (example, control from control-*Streptomyces,* etc.)

(ns) not statistically significant α=0.05

(a) Statistically significant based on P (T< t) two-tail value on two-sample t-test assuming equal variance, α=0.05

(b) α=0.01

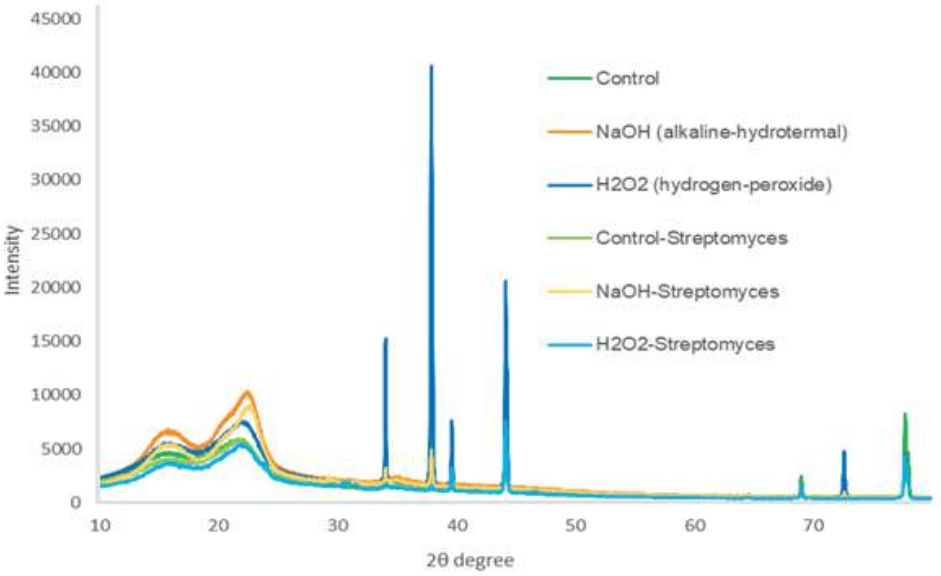

Fig 1 XRD Graph used to measure the crystalline area.

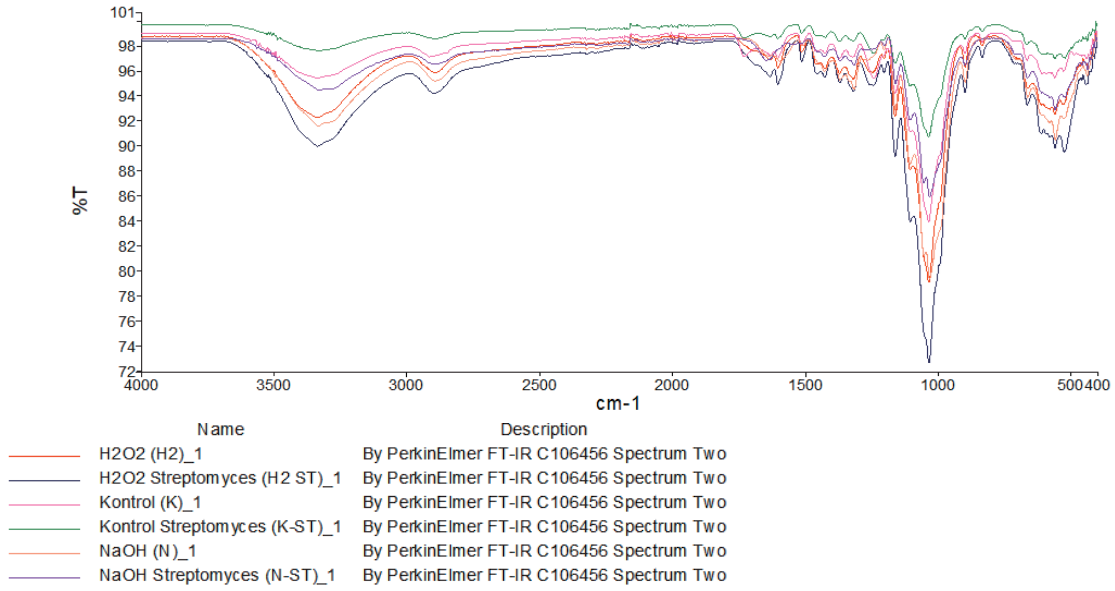

Fig 2 Fourier Transform Infrared Graph.

Table 2 Measurement of reducing sugar and total sugar using different enzyme concentrations (1:2, 1:3, 1:4) also polymerization degree of sugarcane bagasse after four days of enzymatic degradation

Treatment	Enzyme concentration ratio	Reducing sugar (mg ml^{-1})	Total sugar (mg ml^{-1})	Polymerization degree
Control	(1:2)	0.21 ± 0.02	7.64 ± 0.05	37.13 ± 3.22
	(1:3)	0.27 ± 0.02^{ns}	7.41 ± 0.13^{ns}	27.81 ± 1.71^{ns}
	(1:4)	$0.39 \pm 0.01^{b-a}$	$4.24 \pm 0.25^{b-b}$	$10.98 \pm 0.98^{b-b}$
Alkaline-hydrotermal (NaOH)	(1:2)	0.19 ± 0.01^{ns}	4.57 ± 0.07^{b}	24.17 ± 1.44^{a}
	(1:3)	$0.23 \pm 0.03^{ns-ns}$	$4.07 \pm 0.06^{b-a}$	$17.95 \pm 2.33^{a-ns}$
	(1:4)	$0.26 \pm 0.02^{ns-ns-ns}$	$3.52 \pm 0.06^{b-b-a}$	$13.89 \pm 1.39^{a-a-ns}$
Hydrogen-Peroxide (H_2O_2)				
	(1:2)	0.11 ± 0.01^{a}	3.36 ± 0.08^{b}	30.91 ± 2.55^{ns}
	(1:3)	$0.26 \pm 0.02^{ns-a}$	$2.89 \pm 0.15^{b-ns}$	$11.36 \pm 0.52^{b-b}$
	(1:4)	$0.32 \pm 0.01^{a-b-ns}$	$2.67 \pm 0.04^{b-b-ns}$	$8.34 \pm 0.32^{b-b-a}$

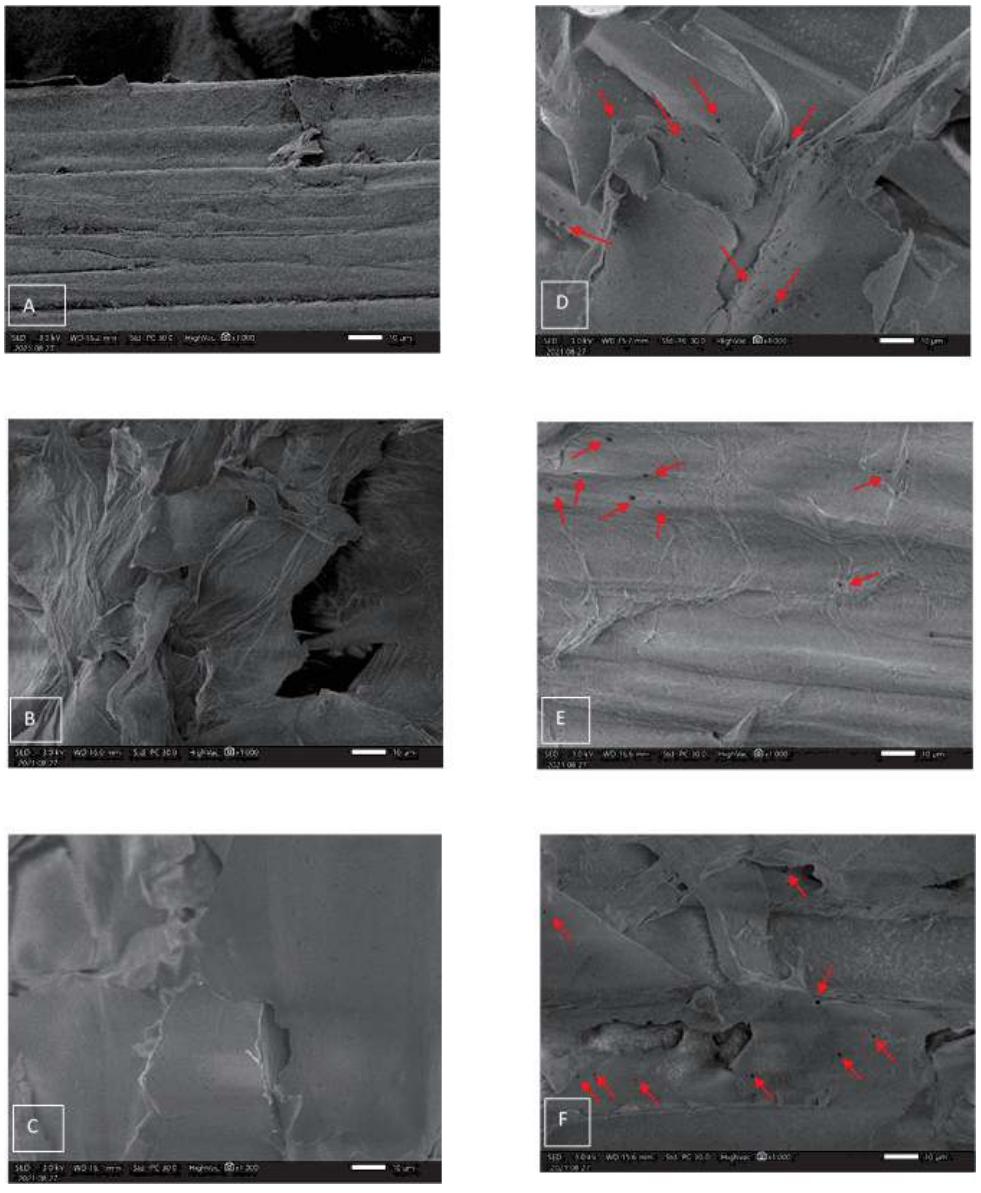

Fig 3 Scanning electron microscope (SEM) photograph. (A). Control (B). Alkaline-hydrothermal (C). Hydrogen peroxide (D). Control-*Streptomyces* (E). NaOH-*Streptomyces* (F). H_2O_2-*Streptomyces*. All photographs shown are at 1000x magnification.

research that use *Cupriavidus basilensis* to degrade acid pretreated rice straw.

Hydrogen bonds in cellulose were cut after both alkaline and peroxide pretreatment, indicated from lower peaks between 3500-3000 cm^{-1}, compared to control. Increase on peak number 3300 cm^{-1} in control-*Streptomyces* and NaOH-*Streptomyces,* except peroxide-*Streptomyces.* Indicates bonds between cellulose were stronger in control-*Streptomyces* and NaOH-*Streptomyces* bagasse, despite the lower cellulose content than its previous treatment. Meanwhile cellulose bonds were weaker in peroxide-*Streptomyces* bagasse compared to peroxide bagasse. Raw bagasse contains ester, indicated from an available peak of 1726 cm^{-1} (Nandiyanto *et al*.2019). All pretreatment weakens these ester bonds.

Raw sugarcane bagasse has higher S (syringyl) compared to (G) guaiacyl lignin, indicated from higher bands around 1300 cm^{-1} compared to around 1200 cm^{-1} (Watkins *et al*. 2015). Miyamoto *et al*. (2018) research also found that most lignin in sugarcane bagasse is syringil lignin. Alkaline pretreatment was only effective to reduce syringil lignin on peak 1300 cm^{-1}. Meanwhile wasn't effective in reducing guaiacyl lignin, indicated from unchanged 1200 cm^{-1} peak. Syringil lignin and guaiacyl bonds remained unchanged when alkaline bagasse was degraded by *Streptomyces* sp S2. Peroxide pretreatment weakens both lower peaks around 1300-1200 cm^{-1}, indicating that peroxide treatment cut both syringil and guaiacyl lignin. *Streptomyces* degradation further weakens both syringil and guaiacyl lignin, indicated from lower 1300-1200 cm^{-1} (Fig 2). This explains the drop in lignin content after peroxide bagasse degraded by *Streptomyces* sp S2, although the final lignin content was similar to control (Table 1).

Hemicellulose identifier bands are stacked with lignin and cellulose identifier bands, between 1200-800 cm^{-1} (Gogna and Goacher 2018). Raw bagasse has hemicellulose that was highly bonded to lignin, indicated from low bands around 1100-900. This result was similar to rice straw in Zulyadi *et al.* (2016) research. Alkaline and peroxide pretreatment was reduce hemicellulose content, correspond to weakened bands between 1100-900 cm^{-1}. Alkaline bagasse hemicellulose content was't changed after *Streptomyces* degradation, indicated from an unchanged peak on 1160 cm^{-1}. However, *Streptomyces* was weakening bonds on 1160 cm^{-1} of peroxide pretreated bagasse. This indicates some of the hemicellulose bonds of peroxide bagasse were cut by *Streptomyces* sp. or remains bonded to lignin, despite an increase in the percentage of hemicellulose (Table 1). Sugarcane bagasse hemicellulose contains β-glycosidic bonds, indicated from a present band on 897 cm^{-1} (Rashid *et al.* 2020) A slight decrease of peak number 832 cm^{-1} was only found in the peroxide-*Streptomyces* bagasse. This indicates *Streptomyces* sp. S2 can decrease p-hydroxyphenyl only after sugarcane is treated with hydrogen-peroxide (Portero- Barahona *et al*. 2019), (Fig 2).

Qi *et al.* research (2018) show similar folding in 4% alkaline (NaOH) pretreated wheat straw. *Streptomyces* sp. S2 was likely to reduce the lignin from the bagasse surface by creating holes and digging out the lignin. This was similar to Xu *et al* (2017) research that used *Cupriavidus basilensis* to degrade rice straw.

The amount of reducing sugar released from degraded sugarcane bagasse was consistent with the crystallinity index value (CrI, Fig 1). This was consistent with Li *et al.* (2016) findings, that higher crystallinity index before saccharification process will increase hexoses sugar that released. The amount of sugar released somehow seems not related to the increase in cellulose percentage after alkaline or peroxide pretreatment (Table 1). Another factor that might affect in decreased hydrolysis efficiency of alkaline pretreated bagasse was higher temperature used (121°C) in alkaline pretreatment than peroxide (60°C), despite shorter time (30 minutes of alkaline vs 90 minutes of peroxide pretreatment). Higher temperature used in alkaline pretreatment makes oligomers such as cellobiose was decomposed faster than glucose yield rate (Mohan *et al*. 2015). Alkaline pretreatment alone produce higher amount of acetic acid, compared to alkaline-peroxide pretreatment. Higher acetic acid inhibits reducing sugar production (Li *et al.* 2016). These result suggest hydrogen-peroxide pretreatment using lower temperature within optimum time can be applicable in fermentation process within pilot plant or industrial settings, since produce higher reducing sugar amount.

In conclusion, hydrogen-peroxide pretreated bagasse was the best substrate to degraded by *Streptomyces* sp S2. Hydrogen-peroxide can weaken both syringil and guaiacyl lignin in bagasse. Which causes lower lignin content when peroxide bagasse is further degraded by *Streptomyces* sp S2. *Streptomyces* sp also increase cellulose and hemicellulose content of peroxide bagasse. These factors affect hydrogen-peroxide bagasse to have the lowest polymerization degree and most effectively hydrolyzed when treated

with *Streptomyces* sp S2.

COMPETING INTEREST

The authors declare that they have no competing interest.

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