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The Effectiveness of Physical and Alkali Hydrothermal Pretreatment in Improving Enzyme Susceptibility of Sweet Sorghum Bagasse

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Abstract

Sweet sorghum bagasse (SSB) obtained after juice extraction is a potential feedstock for fermentable sugars production that can be further fermented to different kinds of products, such as ethanol or lactic acid. The proper particle size resulted from phsyical pretreatment and different pretreatment processes including water, alkali, hydrothermal, and alkali hydrothermal for improving enzyme susceptibility of SSB have been investigated. After grinding to particle sizes of <250 µm, 250-420 µm, and, > 420 µm the sweet sorghum bagasse was washed to eliminate residual soluble sugars present in the bagasse. Dosages of cellulase enzyme used in saccharification were 60 and 100 FPU/g substrate, respectively. The results showed that SSB with particle sizes of 250-420 µm had the highest cellulose (38.33%) and hemicellulose content (31.80%). Although the yield of reducing sugar of 250-420 µm size particles was lower than that of smaller particle (<250 µm), the former was more economical in the energy consumption for milling process. The yields of reducing sugar obtained from enzymatic hydrolysis of alkali hydrothermal pretreated sweet sorghum bagasse were 1.5 and 0.5 times higher than that from untreated sweet sorghum bagasse at enzyme loading of 100 and 60 FPU/g substrate, respectively. Furthermore, alkali hydrothermal pretreatment was able to remove as much as 85% of lignin. Morphological analysis using SEM (Scanning Electron Microscope) showed that samples treated with alkali hydrothermal have more pores and distorted bundles than that of untreated sweet sorghum bagasse. Meanwhile, XRD (X-ray diffraction) analysis showed that pretreated samples had a higher crystallinity and smaller crystallite size than untreated sweet sorghum bagasse, which might be due to removal of amorphous lignin components.

INTRODUCTION

Sweet sorghum (*Sorghum bicolor* L Moench) is one of cereals that has an efficient photosynthetic process, that also produces biomass and sugars. Sweet sorghum also is resistant to drought and requires simple treatments during plantation (Banerji et al., 2013). The juice extraction step of sweet sorghum produces lignocellulosic by product as solid residue or bagasse that could be used as raw material of 2nd generation bioethanol. However, the conversion of lignocellulosic materials into bioethanol is still considered inefficient, due to the difficulties of complex lignin, cellulose and hemicellulose separation. Sweet sorghum is a potential crop to be cultivated and developed in Indonesia. The national production average is 4,000-6,000 tons/year which is expected to produce bioethanol 4,000-7,000 liters/ha/year (Pabendon et al., 2012).

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Results of economic analysis also showed that the production of ethanol from sweet sorghum bagasse (SSB) was more profitable than burning it to be used as an energy source (Gnansounou et al., 2005). Unfortunately, study on the utilization of SSB for producing fermentable sugars or bioethanol in Indonesia was still limited (Pabendon et al., 2012).

The important step in the utilization of lignocellulosic biomass into commercial products is by using the most effective pretreatment to eliminate lignin and hemicellulose from the complex structure of lignocellulose. The purposes of pretreatments are to remove or eliminate lignin and/or hemicellulose, decrease cellulose cristallinity and increase the biomass surface area. In addition, it is necessary to increase the enzymatic susceptibility of cellulose to maximize the yield of sugar production (Gao et al., 2013; Liyakathali, 2014). The existence of lignin and hemicellulose in lignocellulosic materials becomes an obstacle for enzyme susceptibility of the biomass, thus affecting the efficiency of hydrolysis (Liyakathali, 2014).

Particle size reduction (physical pretreatment) is a necessary preliminary step in order to obtain adequate yield in the production of fermentable sugar from lignocellulosic biomass by the available pretreatment methods (Cadoche & Lopez, 1989). Particle size reduction could increase the effective surface area and improve enzyme susceptibility to substrates (Yeh et al., 2010). However, the milling of biomass requires energy input and more capital cost, and thus makes the process non economical. Therefore, it is important to select the proper particle size of biomass which has enough surface area and requires less energy input for grinding (Chen, 2011). The SSB needs milder pretreatment techniques than those other dedicated energy crops and stover, because the cell walls of the SSB have been already broken during the juice extraction step (Choudhary et al., 2012).

Several research groups have already reported their work on SSB pretreatment, such as alkali pretreatment (Wu et al., 2011), hydrothermal treatment (Rohowsky et al., 2013) and low dilute acid (Zhang et al., 2011; Banerji et al., 2013). Alkali pretreatment was considered as one of successful method for pretreating lignocellulose materials. The most widely used alkali soultion for pretreatment is sodium hydroxyde (NaOH). It can remove lignin and hemicellulose in the biomass partially by fracturing the ester bonds, thereby increasing the porosity of the biomass (Cao et al., 2012; Zhang et al., 2011). The efficiency of lignin removal in this pretreatment depends on the type of biomass used (Klinke et al., 2002; Mutepe, 2012). Furthermore, alkali pretreatment does not cause the formation of inhibitor components. Hydrothermal pretreatment is one of the potential pretreatment for increasing cellulosic digestibility and producing fermentable sugars with few inhibitors. However, there are also some weaknesses of hydrothermal pretreatment, for example, it only removes some hemicellulose and imperfectly degrades the lignin-hemicellulose matrix (Long et al., 2014).

Therefore, the aims of this study were to find proper particle size and suitable pretreatment process through a combination of alkali (NaOH) and hydrothermal pretreatment with autoclaving for improving enzyme susceptibility of SSB. The changes in crystallinity and morphology associated with size reduction and alkali pretreatment were also discussed. In addition, this study is expected to produce high fermentable sugars from SSB with enzymatic hydrolysis, as well as to increase the diversity of alternative lignocellulosic material in developing fermentable sugar products.

METHODS

Materials

Sweet sorghum bagasse was obtained from PT. Samirana Surya Semesta, variety of G19 and G20, harvested at 100 days old. All chemicals used were analytical-grade chemicals, such as: hydrogen peroxide 30%, NaOH, ethanol, benzene, dinitrosalicylic acid (DNS) and few of reactants for analysis of material components. Crude cellulase enzyme was used for enzymatic hydrolysis.

Pretreatment and Enzyme Susceptibility

Physical Pretreatment

Dried sweet sorghum bagasse was given physical pretreatment in the form of size reduction by grinding using a ring flaker, hammer mill and disc mill and fractionated using sieves in a sieve shaker. The fractions obtained had particle sizes less than 250 μm m, between 250 and 420 μm and more than 420 μm. The three fractions of SSB were washed with boiled water (1:10) three times to eliminate residual soluble sugars (Cao et al., 2012), then they were dried at 105 °C to constant weight (± 4 hours) (Wang et al., 2012; Yu et al., 2012) and were stored in sealed plastic bags at room temperature. Alkali hydrothermal pretreatment and enzymatic hydrolysis of SSB having the above particle sizes were performed for supporting data to select the proper particle size of SSB.

Alkali Hydrothermal Pretreatment

The washed SSB was pretreated using alkali hydrothermal method referred to Cao et al. (2012). A total of 100 ml of 2% (w/v) NaOH was added into 10 g of dried SSB in 500 ml Duran bottle. The mixture of bagasse and 2 % (w/v) NaOH were stirred for 5 minutes, and autoclaved at 121 °C for 60 minutes. As comparison, alkali hydrothermal was conducted in several conditions, including control (water only), alkali pretreatment without hydrothermal (alkali pretreatment), and hydrothermal pretreatment without alkali solution (hydrothermal pretreatment). After each pretreatment, the reaction was stopped by dipping the Duran bottle in an ice bath. The solid and liquid fractions were separated by filtration. The solid fraction wash washed with distilled water until neutral. A small part of pretreated bagasse was dried at 60 °C for 1 day for fiber analysis, whilst the other part was put in sealed plastic bags and stored in a freezer.

Enzyme Susceptibility Analysis (Enzymatic Hydrolysis)

The effectiveness of sweet sorghum bagasse pretreatment on the cellulase enzyme susceptibility was determined referred to NREL (National Renewable Energy Laboratory) TP-510-42629 (Selig et al., 2008) by using commercial cellulase. In this study, the enzymatic hydrolysis process was not designed to maximize the sugar release but to augment and identify the effectiveness of pretreatment that determined by enzyme susceptibility. Therefore, cellulase was added in excess loading (60 FPU/g substrate and 100 FPU/g susbtrate). Excessive enzyme dosage was used in optimum condition of temperature and pH, that has been determined previously by using carboxymethyl cellulose (CMC-ase) and filter paper (FP-ase). Enzyme activity of cellulase was measured with Filter Paper Activity (FP-ase) method (Adney & Baker, 2008) and CMC-ase method (Ghose, 1987), that is 8.10 FPU/ml dan 7.51 CMC/ml, respectively. Enzymatic hydrolysis was performed in shaking incubator at 50 °C, 150 rpm for 48 hours. Hydrolysis product was analyzed for its reducing sugar content, referred to DNS method (Miller, 1959).

Analysis of Chemical Components and Properties of Materials

Analysis of Chemical Components

Chemical component analysis was performed on sweet sorghum bagasse before and after washing with variations of particle sizes and on sweet sorghum bagasse before and after pretreatment using both physical and hydrothermal alkali pretreatment. Parameters observed including moisture, ash and extractives content using TAPPI (1996), klason lignin content (Sluiter et al., 2011), α-cellulose content (Rowell, 2005) and holocellulose content (Wise et al., 1946).

Proximate analyzes (moisture, ash, crude protein, ether extract, crude fiber and carbohydrate *(by difference)*) were performed on a sweet sorghum bagasse with proper particle size. Proximate analysis of untreated SSB was performed according to AOAC (2005). The crude protein and ether extract content (EE) were analyzed using FOSS equipment (Kjeltec 8400 Analyzer Unit and Soxtec 2050, Hoganas, Sweden). Crude fiber was analyzed using Fibertec apparatus (FOSS Fibertec 2010, Hoganas, Sweden).

Measurement of Crystallinity Index and Cellulose Crystal Size of Materials

Cellulose cristallinity of untreated and pretreated SSB were observed with XRD-700 MaximaX series. Nickel (NI) radiation was filtered with CuKα at wave length 0,15 nm. X-ray was operated at 40 kV and 30 mA. Angle scan was at 2θ 5- 45°. Crystallinity index was calculated based on Zhang et al. (2011). The measurement of cellulose crystal size was using diffraction pattern at lattice plane in 101,10-1, and 002, which was calculated based on Focher et al. (2001).

Morphological Analysis

The morphology of sweet sorghum bagasse was characterized with SEM Hitachi TM-3030. Samples were coated with specific gold, and then being scanned on 5 kV and 500 x of magnification.

Analysis of Reducing sugars

Determination of Reducing Sugar Yield

The reducing sugar content was calculated based on dry weight of the initial sweet sorghum bagasse (Equation 1), based on (Fatriasari, 2014). The estimation of polysaccharides conversion is determined by the assumption that only cellulose and hemicellulose produced the reducing sugars as in Equation 2 and 3 (Choudhary et al., 2012).

Reducing Sugar Yield (% initial sweet sorghum bagasse) :

$$= \frac{\text{Total of Reducing Sugar (g)}}{\text{Initial Sweet Sorghum Bagasse (g)}} \times 100 \qquad (1)$$

The Conversion of Maximum Potential Sugar:

$$= (C/100) \text{ x } 1.11) + (H/100) \text{ x } 1.14) \qquad (2)$$

Where:

1.11 = The conversion factor of cellulose into reducing sugar
1.14 = The conversion factor of hemicellulose into reducing sugar
C = Percentage of cellulose content in sweet sorghum bagasse
H = Percentage of hemicellulose content in sweet sorghum bagasse

Polysaccharides Conversion (%):

$$\frac{RSY \times V_{EH}}{W \times X_{max}} \times 100 \qquad (3)$$

where:

RSY is the reducing sugar yield, V_{EH} is volume in enzymatic hydrolysis (L), W is the weight of sample in enzymatic hydrolysis and X_{max} is the conversion of maximum potential sugar

Statistical Analysis

The experimental design used in this study was a Single-Factor Completely Randomized Design, for study of particle sizes of the material at the physical pretreatment and for study of comparison between the alkali hydrothermal pretreatment and other pretreatments (control, alkali and hydrothermal pretreatment). Data were analyzed for statistical significance by a one-way analysis of variance (ANOVA) to determine individual and interactive effects. Duncan's multiple range tests were performed to test differences among treatments. A 95% confidence level ($p = 0.05$) was applied to all analyses. Software used for statistical analysis was STAR (Statitical Tool for Agricultural Research). The responses or dependent variables of both pretreatment experiments are lignin and hemicellulose contents, as well as enzyme susceptibility which was indicated by the production of reducings.

RESULTS AND DISCUSSIONS

Physical Pretreatment

Effects of Physical Pretreatment and Washing Treatment on Chemical Component of SSB

The physical pretreatment for reduction of SSB particle size used in this study were ring flaker, hammer mill and disk mill. The results of enzymatic hydrolysis of biomass ground by the disk mill was more effective than that ground by the hammer mill because the reduction in size using a hammer mill would form a fiber bond (Zhu et al., 2009). After that, the SSB was fractionated using sieves in a sieve shaker into three fractions. It is important to select the proper particle size of biomass which has enough surface area and requires less energy input for grinding (Chen, 2011). Besides grinding, there was another physical pretreatment used in this study, which was washing treatment using hot water. The aim of this treatment was for eliminating substantially residual soluble sugars present in the stalk after juice extraction thus the sugars obtained in the subsequent process were from the real sugar sources in the bagasse. The chemical components of sweet sorghum bagasse of various particle sizes are presented in Table 1.

Analysis of variance ($p = 0.05$) showed that particle size, washing treatment and interaction between washing treatments and particle size had a significant effect on the extractives of SSB. The losses of chemical components (Figure 1) showed that the component mostly affected by physical pretreatment and washing treatment was extractives. The extractive content decreased 75-88% in all samples of different particel sizes (Figure 1). Extractives are non structural components such as sugars, fats, oils and proteins, that potentially inhibit enzymatic hydrolysis, so that the more the extractive substances removed from the biomass or substrates, the more substrates were hydrolyzed. Moreover, the presence of extractive components in the material will make the lignin condensed, so that lignin becomes difficult to remove. The presence of lignin is an inhibiting factor in enzymatic hydrolysis (Fatriasari, 2014).

The SSB with particle size of 250-420 μm had the highest cellulose and hemicellulose content. The cellulose and hemicellulose was significantly ($p = 0.05$) affected by particle size and washing treatment. Interestingly, the smallest fraction of biomass (<250 μm) in this study did not contain the

Table 1. Chemical components of washed SSB of various particle sizes

Particle Sizes (µm)	**Chemical Components (%)**			
	Extractives	**α-cellulose**	**Hemicellulose**	**Klason Lignin**
< 250	2.07^{a*}	33.53^{c}	30.74^{c}	18.35
250-420	1.59^{a}	38.33^{a}	31.80^{a}	18.15
> 420	1.91^{a}	37.61^{b}	31.46^{b}	17.53

* Values in the same column having the same letter were not significantly different

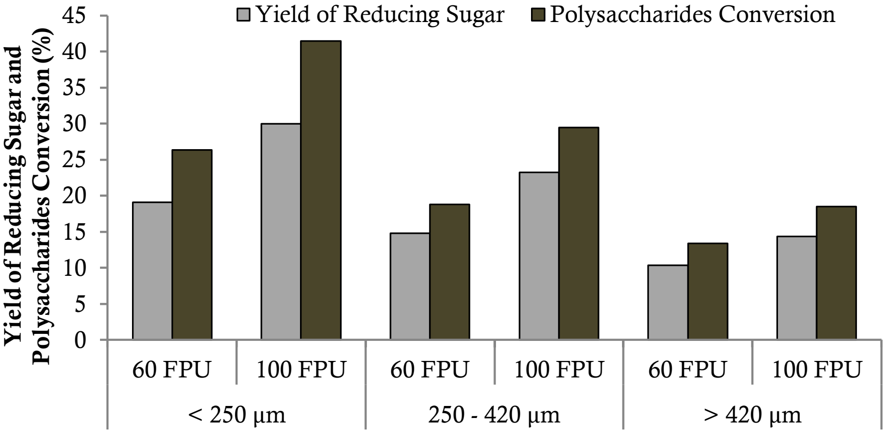

Figure 1. Effects of physical pretreatment and washing treatment on chemical component loss of SSB

highest cellulose. It might be affected by the higher extractive content in the sample of this particle size. In addition, the loss of extractives from SSB with particle size less than 250 µm was lower than that from SSB with particle size 250-420 µm. It probably caused an ineffective delignification that made the cellulose and hemicellulose content in SSB with particle size less than 250 µm lower than those in SSB with particle size 250-420 µm.

Analysis of variance ($p = 0.05$) showed that particle size did not have significant effect on the lignin content. This is consistent with the results of a study conducted by Lamsal & Madl (2011) that found that differences in particle sizes had not have significant effect on lignin content of wheat straw, wheat hull and soybean. On the other hand, the results of analysis of variance ($p = 0.05$) showed a significant effect of washing treatment on lignin content. The washed SSB was used for the subsequent study, so that the residues of sugars from juice extraction had been removed. In addition, it was expected that more substrate was available for the hydrolysis process since the extractives have been removed by washing treatment.

Effects of Physical Pretreatment on Enzymatic Hydrolisis of Washed SSB

The study on the effects of physical pretreatment on cellulase enzyme susceptibility was conducted with alkali hydrothermal pretreatment in the various sizes of SSB prior to enzymatic hydrolysis. The effectiveness of pretreatment in delignification was measured through reducing sugar from enzymatic hydrolisis process. Sun & Cheng (2002) describe that enzymatic hydrolysis of cellulose consists of three steps: adsorption of cellulase enzymes onto the surface of the cellulose, the biodegradation of cellulose to fermentable sugars, and desorption of cellulase.

Reducing sugar content and polysaccharides conversion after enzymatic hydrolysis of the alkali hydrothermal pretreated SSB are shown in Figure 2. Analysis of variance ($p = 0.05$) showed that enzyme loading and particle size had significant effect on reducing sugar yield per initial biomass. The results showed that there was an increase in reducing sugar yield along with the smaller particle size at each dosage of enzyme. The reducing sugar yield was calculated based on the initial weight of biomass. The highest reducing sugar yield was obtained from the sorghum bagasse with particle size less than 250 µm with 60 FPU/g substrate and 100 FPU/g substrate of enzyme loadings, which were 19.06% and 29.95% per initial biomass, respectively. It indicated that the higher the enzyme loading, the more substrates can be converted by the enzyme. However, the use of too much enzyme will result in increase of production costs. The maximum potential sugar that can be

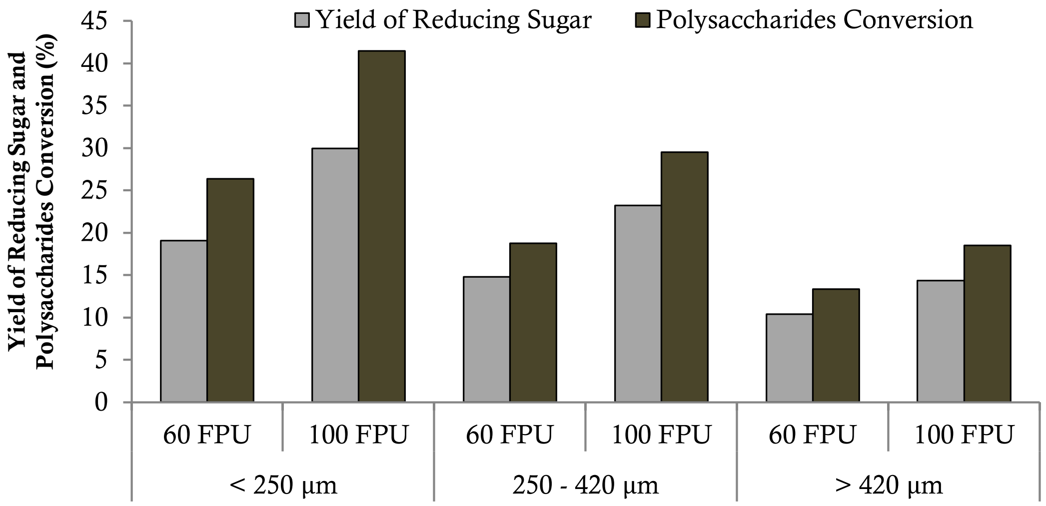

Figure 2. Yield of reducing sugar and polysaccharides conversion of SSB with various particle sizes

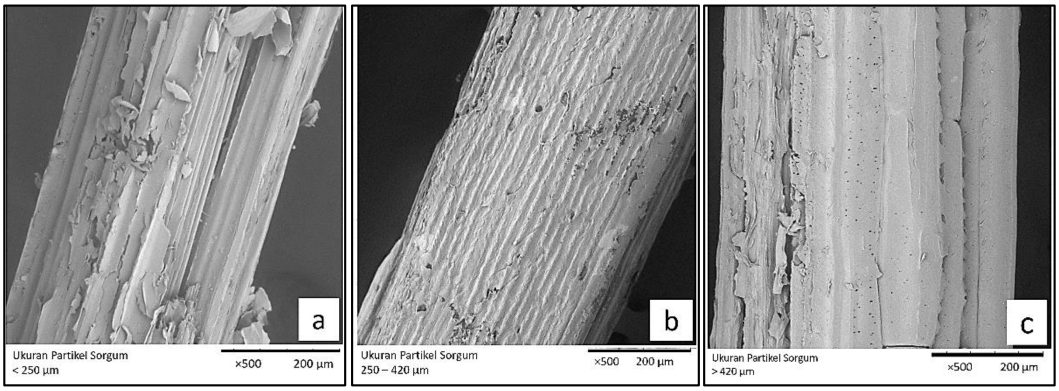

Figure 3. SEM images of SSB with various particle sizes (a: size <250 µm, b: size 250-420 µm, c:size >420 µm)

produced from SSB with particle size less than 250 µm was 72.26 %, as much as 37.22% from cellulose and 35.04% from hemicellulose. Thus, the polysaccharides conversion of SSB in this study reaches 26.38% when enzyme loading was 60 FPU/g substrate and 41.45% when enzyme loading was 100 FPU/ g substrate.

This study shows that cellulose content in SSB of different particle sizes did not correlate with the yield of reducing sugars after enzymatic hydrolysis. SSB with smaller particle size produce the highest reducing sugar yield, while the highest cellulose content was in SSB with particle size of 250 - 420 µm. This indicated that the enzyme is more easily and selectively bound to the outer surfaces of smaller particles, due to the larger contact surface area. This made the hydrolysis process was running more intensively. This result was similar with the results reported by Lamsal & Madl (2011) who found that there was a correlation between particle size reduction and the increase of reducing sugars in soybean hulls, even though the higher cellulose content was in the biomass with larger particel size.

Effects of Physical Pretreatment on SSB Morphology

The results of morphological observation on the surface of SSB are shown in Figure. 3. The observation clearly showed that there were pores in SSB with diffferent particle sizes, with more pores seen in that with smaller particle size (<250 µm). Therefore, the enzymes can penetrate the SSB with smalller particle size more easily. Previous study Chen (2011) reported that sorghum bagasse of particle size 1-2 mm had larger porosity than the one of 9.5-18 mm, because the small particles not only have larger specific surfaces but also may have lower crystallinity and lower degree of polymerization due to milling (Khullar et al., 2013). The cell walls of SSB were broken because of pressing of sweet sorghum juice out from the stems. Juice extraction causes breakage of cell walls and

Table 2. The crystallinity index and crystallite size of cellulose at different sizes particle of SSB

Particle Size	**Crystalline**	**Amorphous**	**CrI (%)**	**Crystallite Size (nm)**		
				D (002)[a]	**D (101)**[b]	**D (10-1)**[b]
< 250 µm	0.27	1.07	20.39	7.36	32.24	40.00
250-420 µm	0.35	0.99	26.18	7.84	15.82	20.23
> 420 µm	0.31	1.26	20.03	7.17	10.23	26.26

[a] D (002) is lattice plane of crystalline.

[b] D (101) and D (10-1) is lattice plane of amorphous.

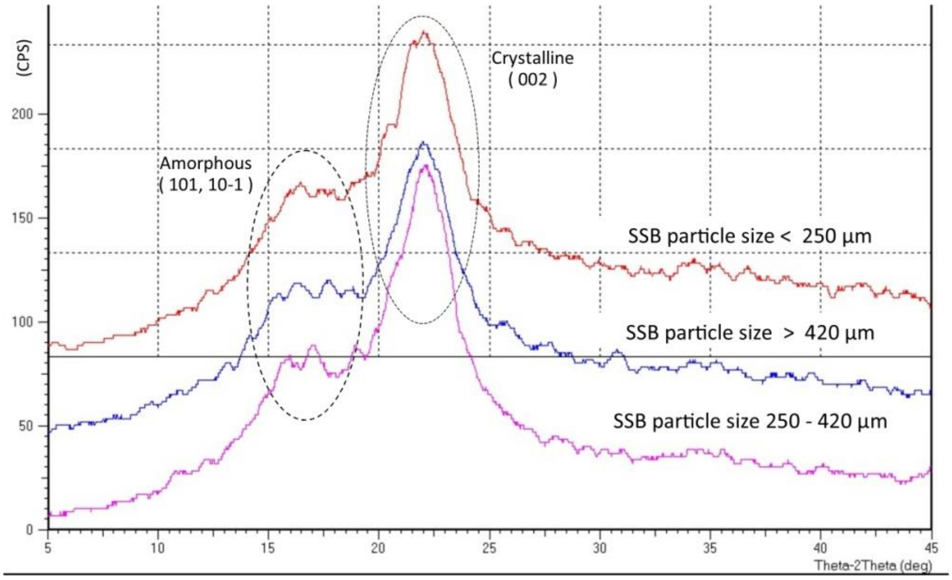

Figure 4. XRD spectra of sweet sorghum bagasse with various particle sizes

thus the pretreatment required for SSB is expected to be less intense than that required for other feedstocks (Choudhary et al., 2012).

Effects of Physical Pretreatment on Crystallinity of SSB

Crystallinity Index (CrI) represents relative amount of crystalline and amorphous part of a material that can be measured by X ray diffraction method. The increase of crystallinity index affected by the increase of crystaline part over the amorphous part (Fatriasari et al., 2014). The calculated crystallinity index and crystallite size of different particle size of SSB are given in Table 2. The SSB with particle size between 250 and 420 µm has the highest crystallinity index. This correlated with the highest loss of amorphous parts (hemicellulose and lignin) from SSB due to washing treatment (Figure 1). Thus, the phenomenon was most likely due to the loss of these amorphous part of biomass rather than due to the changes in the crystalline structure of cellulose. This was also confirmed by other researchers, who stated that removal of noncrystalline parts such as hemicellulose and lignin can increase the crystallinity of the entire biomass (Kim et al., 2013). Results of this study also showed that CrI did not have significant effects on reducing sugar yields after enzymatic hydrolysis, which was in agreement with Gao et al. (2013). Nevertheless, Yeh et al., (2010) reported that reduction in the cellulose crystallinity upon mechanical pretreatment (*i.e.*milling) was a possible cause behind the improved hydrolysis.

The largest crystallite size at lattice plane (002) was found in the particle size between 250 and 420 µm (Table 2). Yeh et al. (2010) reported that the crystalline region described as the lattice plane 002 of XRD diffraction ranged from 2θ of 22 up to 23°, while the amorphous portion of the lattice plane 101 was at 2θ between 15 and 18°. The crystallite size obtained in this study was larger than that studied by Zhang et al. (2011), which was 4.5 nm, using an 18 mesh sweet sorghum bagasse. In addition, Focher et al. (2001) obtained a crystallite size of 3.6 nm in sorghum fiber, but they did not explain the size of the sorghum fiber particles used. Therefore, it can be concluded that the crystallite size as well as CrI of SSB did not have effects on the performance of enzymatic hydrolysis.

Table 3. Chemical components of untretaed and pretreated SSB

Pretreatments	Hemicellulose (%)	Klason Lignin(%)	Yield (%)
Untreated	31.80 ^{b*}	18.15 ^{ab}	-
Water	34.61 ^{a}	17.54 ^{bc}	95.68
Alkali	29.12 ^{c}	16.92 ^{c}	92.20
Hydrothermal	31.69 ^{b}	18.26 ^{a}	84.99
Alkali Hydrothermal	29.88 ^{c}	4.35 ^{d}	64.76

* Values in the same column having the same letter were not significantly different.

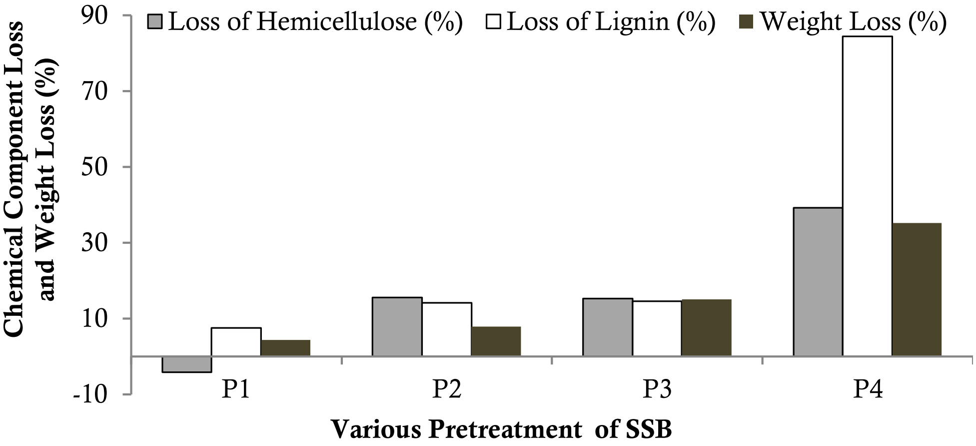

Figure 5. Chemical component loss and weight loss of SSB exposed to various pretreatments (P1: water, P2: alkali, P3: hydrothermal, P4: alkali hydrothermal)

Figure 4 shows that primary peak (002) at particle size less than 250 μm was slightly different from those at other sizes. There is also a slight shift of 2θ angle in the secondary peak (101). It indicated that physical pretreatment does not alter the cellulose structure, because the amorphous and crystalline regions of cellulose were still emerged, even though there was a slight shift of the 2θ angle. In addition, all peaks in the XRD spectra show quite flat diffraction patterns. This suggested the presence of highly amorphous cellulose (Zhang et al., 2011), although the spectrum of SSB with particle size between 250 and 420 μm looks more wavy than others. This is in accordance with the higher crystallinity index and crystallite size of cellulose in SSB with particle size between 250 and 420 μm.

Although the use of SSB with particle size less than 250 μm resulted in better performances (reducing sugar yield, morphological structure and CrI) than that of between 250 and 420 μm, the latter was chosen for the subsequent study, because there was another thing that was considered, energy consumption for grinding the biomass. There will be much more energy needed to grind the biomass to less than 250 μm than to larger particle sizes. Cadoche & Lopez (1989) calculated the energy consumption for size reduction of wheat straw using hammer mill.The energy consumption was 21 kWh/tonne and 42 kWh/tonne for particle size 3.2 mm, and 1.60 mm, respectively. It indicated that grinding of biomass to a smaller particle size consumed much more energy than that to larger ones. Moreover, the highest cellulose content was found in SSB with particle size of 250-420 μm, so that it could be a more potential substrate for the production of fermentable sugars.

Alkali Hydrothermal Pretreatment

Effects of Alkali Hydrothermal Pretreatment on Chemical Components of Sweet Sorghum Bagasse

The results of proximate analysis of washed SSB with 250-420 μm particle size show that it contains moisture, ash, crude protein, ether extract, crude fiber and carbohydrate *(by difference)* as much as 7.07; 3.02; 0.96; 0.41; 31.30 dan 95.61% on dry weight basis, respectively. It is similar with proximate analysis of SSB cultivated in Iran, conducted by Almodares et al. (2009) who got 5.40% crude protein and 40.60% crude fiber. Mutepe (2012) reported the results of proximate analysis of four SSB varieties, which contained protein 1.51-2.65%, ash 2.90 - 4.42%, and fat 0.37-

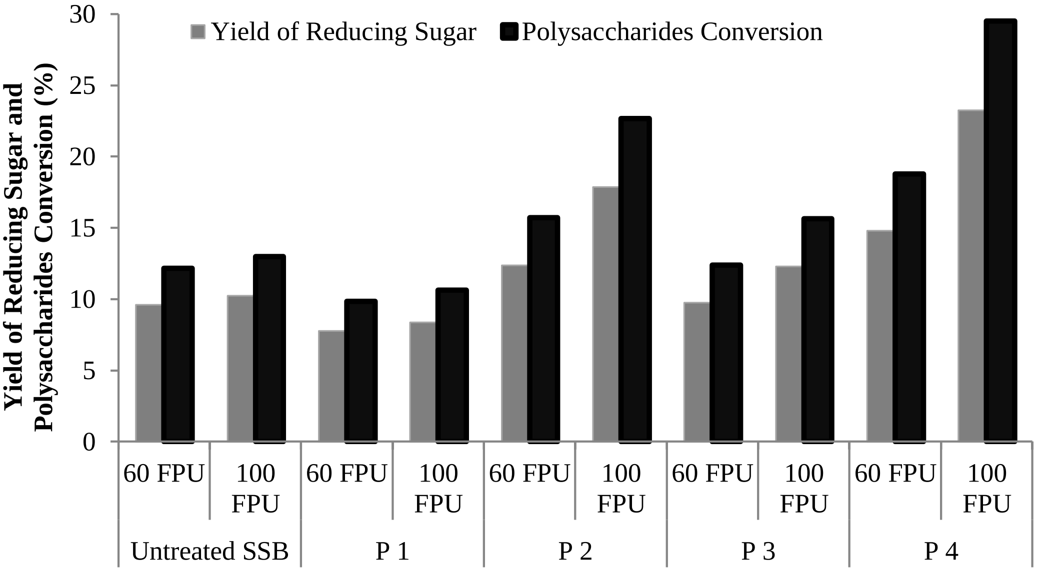

Figure 6. Yield of reducing sugar and polysaccharides conversion on various pretreatments (P1: water, P2: alkali, P3: hydrothermal, P4: alkali hydrothermal)

0.54%. All of these results indicated the varieties of SSB cultivated in Indonesia were similar with varieties in other countries, although the differencies of composition of SSB might be influenced by the type of cultivar, soil type and environmental factors (Mutepe, 2012). The percentage of crude fiber represented carbohydrate fraction which was insoluble in dilute acid or alkaline. In this study, the percentage of crude fiber was lower than total fiber fraction (Table 1), because some cellulose and hemicellulose were soluble in dilute acid used during the analysis of crude fiber.

The fiber fraction content of SSB used in the alkali hydrothermal pretreatment is shown in Table 1. This composition of SSB is in agreement with that reported by Choudhary et al. (2012) , who used SSB that was harvested in the USA, with the composition of cellulose, hemicellulose and lignin 36.9, 17.8, and 19.5%, respectively. Although the percentage of cellulose in this study was lower than the data reported previously, it was still has a high cellulose content, which is very promising to be used as substrate for the production of ethanol or fermentable sugars. The effectiveness of pretreatment methods can be evaluated by observing the changes of material compositions, removal of hemicellulose and lignin, reduction of cristallinity of cellulose, and dry matter loss (Kumar et al., 2009; Cao et al., 2012). The changes of chemical compositions of SSB due to alkali hydrothermal pretreatment are shown in Table 3.

The analysis of variance showed that there was no significant differences (*p*=0.05) in hemicellulose content between alkali pretreatment and alkali hydrothermal pretreatment, but there was a significant difference in lignin content between the two pretreatments. Thus, the use of high temperature with alkali (NaOH) in pretreatment could accelerate the removal of lignin from the biomass as also reported by Wu et al. (2011). Hendriks & Zeeman (2009) explained that the first reactions during alkali pretreatment are solvation and saphonication. This causes a swollen state of the biomass and makes it more accessible for enzymes and bacteria. Sodium hydroxide effectively attacks the linkages between lignin and hemicellulose in lignin-carbohydrate complexes (Kim et al., 2016).

Alkali hydrothermal pretreatment could remove as much as 39.15% of hemicellulose from SSB. (Figure 5). The analysis of variance and Duncan's multiple range tests showed that there were significant differences (*p*=0.05) in lignin content between alkali hydrothermal pretreatment and other pretreatments used in this study. The alkali hydrothermal pretreatment could remove as much as 85% of lignin from SSB (Figure 5). Lignin removal increases enzyme effectiveness by eliminating nonproductive adsorption sites and by increasing access to cellulose and hemicellulose (Kumar et al., 2009). This result was similar with that reported by Cao et al. (2012), who used combination of alkali pretreatment and liquid hot

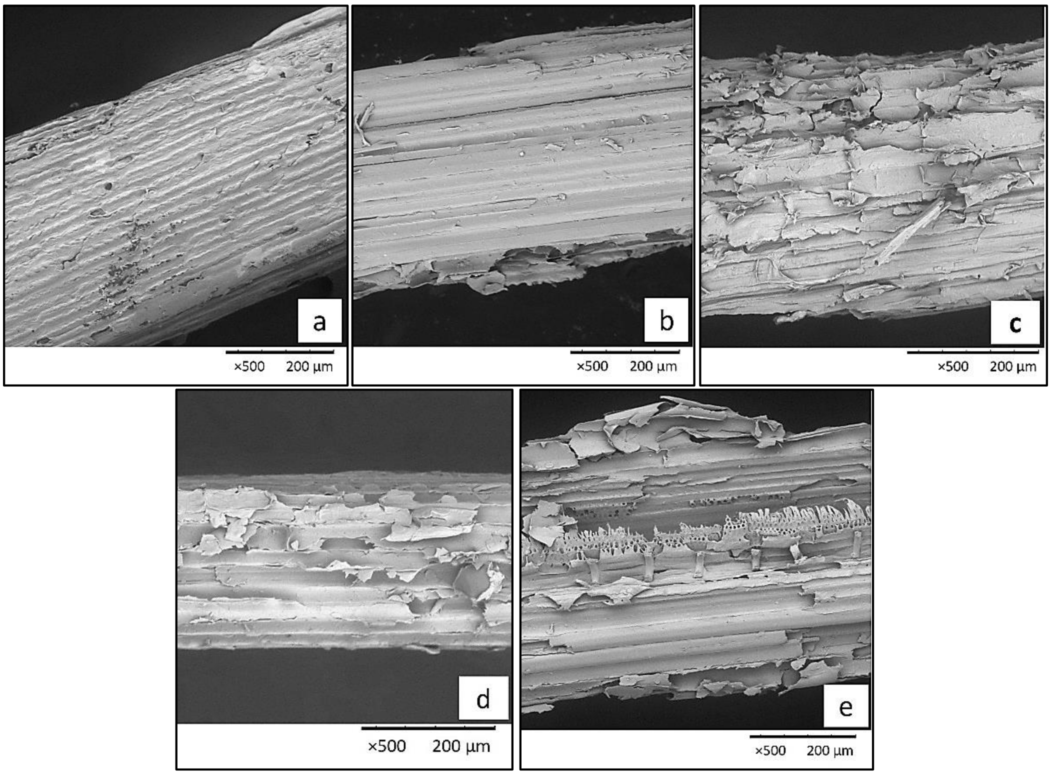

Figure 7. SEM images of SSB (a: untreated, b: water pretreatment, c: alkali pretreatment, d: hydrothermal pretreatment, e: alkali hydrothermal pretreatment)

water that could remove 84% of lignin from sugarcane bagasse. Meanwhile, the result of study conducted by Toquero & Bolado (2014) using wheat straw showed that alkali hydrothermal pretreatment removed 51% of acid insoluble lignin. These suggested that the performance of pretreatment for different types of lignocellulose could be differ widely depending on the composition of biomass and severity of pretreatment conditions, although under same chemical pretreatment (Umagiliyage et al., 2015).

The weight loss of biomass caused by alkali hydrothermal pretreatment was the highest than those caused by other pretreatments used in this study. However, it only caused significant reduction to both lignin and hemicellulose content, but retained the cellulose (Figure 5). Cao et al. (2012) reported that high temperature and pressure during alkali pretreatment will be beneficial for retaining cellulose, removing hemicellulose and lignin, but not good for retaining dry matter.

Effects of Alkali Hydrothermal Pretreatment on Enzymatic Hydrolisis

The results of the analysis of variance (p = 0.05) test showed that the pretreatment and the enzyme loading did not affect the yield of reducing sugar. The results of enzymatic hydrolysis of untreated and pretreated of SSB are shown in Figure 6. The highest reducing sugar yield was obtained in alkali hydrothermal pretreatment, that was 14.79% with enzyme loading of 60 FPU/g substrate and 23.24% with enzyme loading of 100 FPU/g substrate. It can be confimed that the use of relatively high temperature and pressure during pretreatment were better for cellulose hydrolysis than that of ambient temperature and normal pressure. Furthemore, alkali autoclaving affected biomass porosity and internal surface, due to high removal of lignin and partial solubilization of xylan side chains (Kumar et al., 2009).

Maximum potential sugar that could be obtained according to equation 2 was 78.80%. Therefore, the polysaccharides conversion of the alkali hydrothermal pretreatment were 18.77% (enzyme loading of 60 FPU/g substrate) and 29.49% (enzyme loading of 100 FPU/g substrate). On the other hand, the yield of reducing sugar obained from hydrothermal pretreatment with enzyme loading of 60 FPU/g substrate was only 9.77g/ 100g SSB, which was similar with that from untreated SSB (9.59g/100 g SSB). It is consistent with the low lignin removal in hydrothermal pretreatment, which hindered cellulose and hemicellulose hydrolysis, therefore, it caused low sugar release since the enzyme could not attack the

Table 4. The crystallinity index and crystallite size of cellulose after different pretreatments of SSB

Pretreatments	**Crystalline**	**Amorphous**	**CrI (%)**	**Crystallite Size (nm)**		
				D (002)[a]	**D (101)[b]**	**D (10-1)[b]**
Untreated	0.35	0.99	26.18	7.84	15.82	20.23
Water	0.47	1.21	27.88	6.61	20.99	28.02
Alkali	0.56	1.28	30.62	6.64	10.76	-
Hydrothermal	0.54	1.30	29.41	6.46	-	9.32
Alkali Hydrothermal	0.68	0.91	42.82	7.14	-	9.12

[a] D (002) is lattice plane of crystalline.

[b] D (101) and D (10-1) is lattice plane of amorphous.

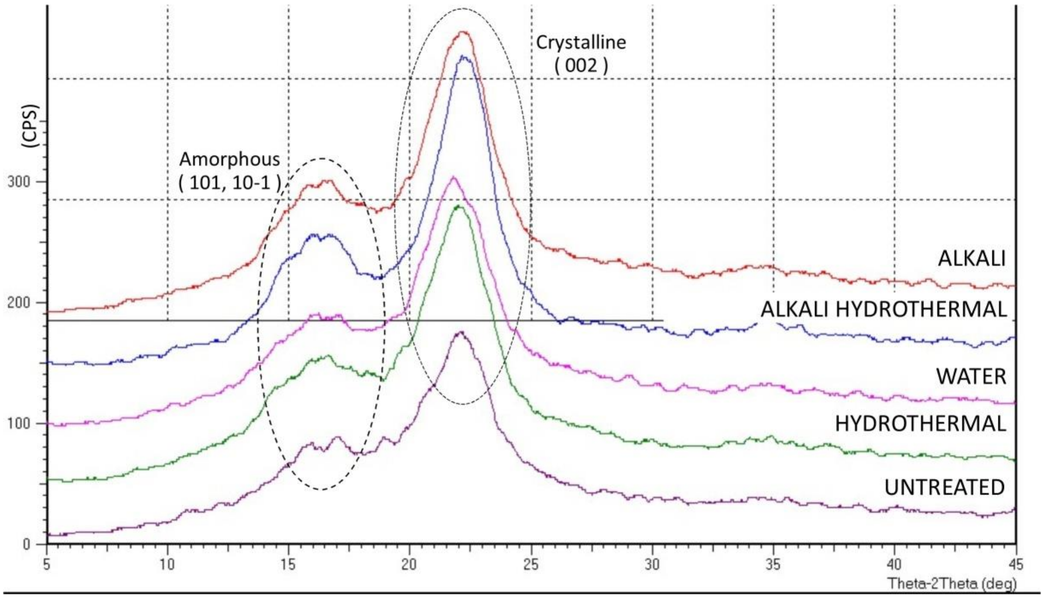

Figure 8. XRD spectra of sweet sorghum with various pretreatments

cellulose effectively (Toquero & Bolado, 2014). The highest total reducing sugars in this research (2.17 g/L) was lower than that reported by Matsakas & Christakopoulos (2013), who obtained 81.9 g/L of sugars in the hydrolysates from SSB pretreated by hydrothermal continued with 12 hours enzymatic hydrolysis (3% substrate and enzyme loading of 10 FPU/g substrate). It might be due to the low activity of cellulase and β-glucosidase used in this research.

Effects of Alkali Hydrothermal Pretreatment on SSB Morphology

The image of untreated SSB exhibits a compact rigid particle structure with little pores and closed holes in which cellulose is closely wrapped by hemicellulose and lignin (Figure 7). It has similarities with SSB exposed to water pretreatment, indicating that water addition did not affect the surface of SSB significantly.

Alkaline addition in pretreatment such as shown in Figure 7 caused the fiber bundles distorted. This is in agreement with the finding of Cao et al. (2012) that showed similar change in surface structure when SSB is delignified by alkaline due to degradation of the lignin and hemicellulose in the bagasse. Pretreatment at high temperatures, such as in alkali hydrothermal and hydrothermal caused the formation of more pores on the surface of biomass, nevertheless the size of pores in the SSB pretreated by the two methods are different. Combination of alkali and high temperature produced the bigger pores than did high temperature pretreatment alone. The bigger pores provide more accesible surface area to cellulase. The results of alkali hydrothermal pretreatment were consistent with the yield of reducing sugar and removal of lignin. The more lignin eliminated, the larger the surface area, so that more cellulose was converted into sugar.

Effects of Alkali Hydrothermal Pretreatment on Crystallinity of SSB

The crystallinity of lignocelluloses has often been suggested as one of the major factors to effect enzymatic digestibility of cellulose (Gao et al., 2013; Kim et al., 2013). Actually, disruption of interchain and intrachain hydrogen bonding of cellulose fibrils can be attributed to the pretreatment processes and leads to decrease in the crystallinity of cellulose (Kim et al., 2013). Table 4 illustrates the CrI of untreated and pretreated of SSB. After pretreatments, the CrI values of pretreated SSB were higher than that of untreated SSB. This condition was similar to that of sugarcane bagasse reported by Gao et al. (2013) and that of sweet sorghum bagasse reported by Wu et al. (2011).

The increase of the CrI values of pretreated SSB were in accordance with the lignin removal of pretreated SSB (Figure 5). It is suggested that the increase in CrI probably was primarily due to the removal of amorphous lignin component rather than due to the decrease of relative amount of crystalline region in cellulose or due to the removal of the amorphous hemicellulose. Other factors that probably affect the CrI were hornification caused by drying process (Fatriasari et al., 2014; Luo & Zhu, 2011) and method of CrI determination.

The crystallite size at lattice plane (002) of the pretreated SSB is smaller than that of the untreated SSB. This is in accordance with Zhang et al. (2011) who reported that the decrease of the crystallite size of pretreated SSB compared to untreatred SSB. Unfortunately, the crystallite size at lattice plane (101) and (10-1) of several pretreated SSB can not be calculated, because there were no value of Full Width at Half Maximum (FWHM) in this peak. The results of calculation of the crystallite sizes are used for comparing crystal sizes among samples, rather than for describing the actual size of the crystal in the samples.

The primary peak (002) and secondary peak (101) of the XRD spectra for alkali hydrothermal and alkali pretreatment had a slight shift in the 2θ angle compared with the peak of the untreated SSB (Figure 8), whereas hydrothermal pretreatment and water pretreatment had almost similar peaks with the spectra peak of the untreated SSB. Liu et al. (2012) reported that the changes in the width of the crystallization peak indicated the intensity transformation in the cellulose molecular hydrogen bonding and this phenomenon would be beneficial for subsequent enzyamatic hydrolysis. However, in this study, the decrease of crystallization diffraction peak width in pretreated SSB was not significant, indicating that some of these pretreatments did not alter the cellulosic structure of the material, especially in the crystalline portion of cellulose, even though there is a slight shift in the 2θ angle. Data in this study also showed that CrI and crystallite size did not correlate with the yield of reducing sugars. Despite the high crystallinity index value and no sign of peak width decrease, the alkali hydrothermal pretreatment resulted in the highest yield of reducing sugars.

CONCLUSIONS

Sweet sorghum bagasse with particle sizes of 250 - 420 μm were the most appropriate for its enzymatic hydrolysis, while alkali hydrothermal pretreatment was considered the most suitable pretreatment, due to high removal of lignin and high reducing sugar yield after enzymatic hydrolisis. Pretreatment causes morphological damage, an increase in the crystallinity index, and a decrease in the size of cellulose crystals in the SSB.

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