Enzymatic saccharification of liquid hot water and dilute sulfuric acid pretreated oil palm empty fruit bunch and sugarcane bagasse

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Abstract. Oil palm empty fruit bunch (OPEFB) and sugarcane bagasse (SB) are potential feedstocks for the production of bioethanol. In this study OPEFB and SB were pretreated by liquid hot water and dilute sulfuric acid (3% H₂SO₄), and continued with enzymatic saccharification. Heating treatment for both methods was conducted in an autoclave at 121 °C for 1 hr. The saccharification was performed up to 72 hours with cellulase enzyme loading of 10, 20, and 30 FPU per g biomass. Results showed that OPEFB and SB pretreated with H₂SO₄ produced higher reducing sugars than those pretreated by liquid hot water. Higher enzyme loading also resulted in higher reducing sugars. Reducing sugars obtained from enzymatic saccharification of OPEFB were higher than those obtained from SB. The highest total reducing sugars (50.48 g/100 g biomass) was obtained from OPEFB pretreated with 3% H₂SO₄ at enzyme loading of 30 FPU per g biomass.

1. Introduction
Oil palm empty fruit bunch (OPEFB) and sugarcane bagasse (SB) are among potential feedstocks abundantly available in Indonesia for producing bioethanol[1,2]. The conversion of the lignocellulosic biomass to ethanol involves the pretreatment, enzymatic hydrolysis of cellulose to simple sugars, and fermentation of the sugars to ethanol.

Since pretreatment of biomass is an important step in the conversion, there have been numerous efforts to pretreat the lignocellulosic biomass, including OPEFB and SB[3-8]. Acidic pretreatment is one of pretreatment methods that can effectively modify the chemical structures of lignocellulose. In this case, acids serve as catalysts to hydrolyze carbohydrates, especially hemicellulose, so that they loosen the lignin-hemicellulose barrier, which can facilitate the penetration of cellulase enzyme to cellulose matrices[9]. Hot water pretreatment is another method of pretreatment that mainly aimed at removing of hemicellulose from lignocellulosic biomass. Compared to dilute acid pretreatment, hot water pretreatment is considered environmentally friendlier and has some advantages, for example lower risk of equipment corrosion, and less xylose degradation, which means lower amount of inhibitors in the hydrolysate [10].In the enzymatic hydrolysis process, enzyme loading is one of factors that affect amount of sugars obtained. The amount of enzyme added to the pretreated biomass should be appropriate, so that the saccharification process is efficient.

The present study was conducted to compare the hot water and dilute acid pretreatment in the production of reducing sugars, both in the hydrolysate of pretreatment and in the hydrolysate of
enzymatic saccharification. Besides that, the effects of enzyme loading on the production of the reducing sugars during enzymatic saccharification were also observed.

2. Materials and Methods
Raw materials used in this study are OPEFB and SB, which were obtained from Sukabumi and PG Rajawali, Subang Indonesia, respectively. The materials were dried and ground into particle sizes 40-60 mesh, then they were kept in sealed plastic bags and stored in a container. All other chemicals used were analytical grade without further purification.

2.1. Chemical component analysis of OPEFB and SB
Moisture content of biomass was determined according to TAPPI Test Method T 264 cm-97[11], while ash and extractive content were determined according to TAPPI Test Method T 211 om-02 12] and TAPPI Test Method T 204 cm-97[13], respectively. NREL LAP 003 [14] was used as a reference for the determination of acid insoluble lignin and Wise method [15] was used for the determination of holocellulose. The α-cellulose content was determined according to Rowell (2005)[16]. Hemicellulose content was obtained from subtraction of holocellulose and α-cellulose.

2.2. Pretreatment of OPEFB and SB
The materials were put in an Erlenmeyer flask. Water or 3% sulfuric acid were added to the flask, so that the solid loading of the suspension was 6%. The Erlenmeyer flask was tightly closed, and put in an autoclave for heat treatment. Heating in the autoclave was performed at 121 °C for 1 hour. After the process completed, the samples were cooled down, and continued with separation between soluble and insoluble fraction by filtration. The insoluble fraction, also called pulp, was neutralized using 1% NAOH and was washed with distilled water. The pulp recovery of the pretreated samples were determined by measuring the weight of the pulp and determination of moisture content of the pulp. Both fractions were kept in a refrigerator for further processes or analyses.

2.3. Enzymatic saccharification of pretreated OPEFB and SB
Enzymatic saccharification of the pretreated OPEFB and SB were conducted according to NREL. As much as 0.3 g of pretreated samples were put in centrifuge tubes. About 15 mL of 0.1 M citrate buffer pH 4.8 was added to the sample, continued with the addition of 300 µL of 2% sodium azide and 10, 20 and 30 FPU of cellulase enzyme (Meicellase from Meiji Sheika, enzyme activity 200 FPU/mL). The citrate buffer was then added to get the total volume of 30 g. The samples were incubated in a waterbath shaker at 150 rpm, 50 °C for 72 hours. Sampling of the hydrolysates were conducted every 24 hours. After the saccharification was completed, the samples were heated in boiled water for 5 minutes to inactivate the enzyme. The samples were kept in refrigerator for further analyses.

2.4. Analysis and determination of reducing sugars
Determinations of reducing sugars in the soluble fraction and in the hydrolysates of enzymatic saccharified samples were performed using dinitro salicylic acid (DNS) method[17]. The absorbance of the DNS preparations were observed using UV-Vis Spectrophotometer (Hitachi-U-2001) at 520 nm. The yields of the reducing sugars from the pretreated samples were determined based on the weight of the initial biomass.

3. Results and Discussions
3.1. Chemical compositions of OPEFB and SB
OPEFB contained lower ash, acid insoluble lignin and hemicellulose than did SB, but higher extractives and α-cellulose (table 1). Based on these compositions, it can be predicted that OPEFB is more potential in producing sugars than is SB. All the chemical contents, except hemicellulose
content of OPEFB and SB used in this study were slightly lower than those used in our previous study [18,19].

Table 1. Chemical compositions of oil palm empty fruit bunch (OPEFB) and sugarcane bagasse (SB).

| Components              | OPEFB | SB     |
|-------------------------|-------|--------|
| Ash (%)                 | 2.91 ± 0.03 | 4.57 ± 0.09 |
| Extractives (%)         | 2.69 ± 0.11 | 2.36 ± 0.06 |
| Acid insoluble lignin (%) | 23.62 ± 0.08 | 25.42 ± 0.15 |
| α-cellulose (%)         | 41.23 ± 0.77 | 36.53 ± 0.18 |
| Hemicellulose (%)       | 26.55 ± 0.57 | 27.04 ± 0.89 |

3.2. Pulp Recovery
The effects of each pretreatment on the pulp recovery (figure 1) show that pretreatment using sulfuric acid resulted in lower pulp recovery than that using only water. These were observed either in pretreated OPEFB or in pretreated SB, and suggested that sulfuric acid degraded more chemical components in the material into lower molecular weight compounds that dissolved in the acid solution and became soluble compounds. These phenomena were confirmed by the results of reducing sugars in the soluble fractions (figure 2). The pulp recovery from pretreated OPEFB was slightly higher than that from pretreated SB, either in that obtained from LHW or from sulfuric acid pretreatment. These indicated that OPEFB was slightly harder to degrade than was SB. The amount of pulp recovered from sulfuric acid pretreatment of OPEFB was comparable with those from hydrogen peroxide enhanced oxalic acid pretreatment process with alkaline impregnation, amounted 60.7-65.2% [3], but much lower than those obtained from fungal pretreatment (85-90%) [18].

Figure 1. Pulp recovery from oil palm empty fruit bunch (OPEFB) and sugarcane bagasse (SB) after pretreatment using liquid hot water (LHW) and 3% sulfuric acid at 121 °C for 1 hour.

Figure 2. Reducing sugars in soluble fraction after pretreatment of oil palm empty fruit bunch (OPEFB) and sugarcane bagasse (SB) using liquid hot water (LHW) and 3% sulfuric acid at 121 °C for 1 hour.
3.3. Reducing sugars from soluble fractions

Hot water and sulfuric acid could hydrolyze polysaccharides in the pretreated biomass as shown in figure 2. However, the acid could better degrade the polysaccharides, which was shown by the much higher reducing sugars in the soluble fractions of sulfuric acid pretreatment than those in the soluble fractions of LHW pretreatment. The catalysis of water in the LHW pretreatment was due to the hydronium ions (H$_3$O$^+$) generated in situ by auto-ionization of water, and acetic acid generated by hydrolysis of hemicellulose[20,21]. This was not as strong as the catalysis of sulfuric acid that could produce hydrogen ions (H$^+$) with low pKa. Figure 2 also shows that there were slightly higher reducing sugars from SB than those from OPEFB in the soluble fractions. This was probably due to the slightly higher hemicellulose in SB than that in OPEFB (table 1).

![Figure 3](image-url)

**Figure 3.** Reducing sugars release after enzymatic saccharification of (a) oil palm empty fruit bunch (OPEFB) and (b) sugarcane bagasse (SB) pretreated by liquid hot water (LHW) (dashed lines) and 3% sulfuric acid (solid lines) at 121 °C for 1 hour with enzyme loading of 10 (□), 20 (△), and 30 (○) FPU/g substrate.

3.4. Reducing sugars from enzymatic saccharification

Data in figure 3a and 3b show that reducing sugars obtained from enzymatic saccharification were increased with increase of enzyme loading and with longer hydrolysis. The data also show that LHW pretreatment produced lower reducing sugars than did sulfuric acid pretreatment. The highest reducing sugars obtained from the pretreatment OPEFB and SB using LHW were 11.86 and 9.67 g/100 g biomass, respectively, while those from sulfuric acid pretreatment were 31.74 and 20.0 g/100 g biomass, respectively. Much higher differences of reducing sugars obtained from LHW and sulfuric acid pretreatment were observed when the total reducing sugars from the soluble fractions were added to those obtained from enzymatic saccharification. As much as 50.48 and 40.41 g/100 g biomass of reducing sugars could be obtained from sulfuric acid pretreated OPEFB and SB, respectively, while there were only 13.59 and 11.73 g/100 g biomass of reducing sugars obtained from LHW pretreated OPEFB and SB, respectively (figure 4a and 4b).Of the two biomass feedstocks used in this study, SB produce higher reducing sugars in the soluble (liquid) fraction (figure 2). However, OPEFB could produce much higher reducing sugars from enzymatic saccharification of the insoluble fraction (figure 3). Therefore, the total amount of reducing sugars from OPEFB was higher than those from SB (figure 4). Compared to our previous study on pretreatment of OPEFB using fungal pretreatment, the
reducing sugars obtained in this study were much higher than those using fungal pretreatment 8.98-13.08% [18]. On the other hand, reducing sugars obtained from acid pretreatment of SB were lower than those obtained from lime pretreatment (17.51-35.69%) and sodium hydroxide pretreatment (29.31-45.69%) [19]. Nevertheless, it would be much easier to recover sugars from soluble fractions of acid pretreated biomass than that of alkaline pretreated biomass.

Figure 4. Total reducing sugars obtained from soluble fractions and from enzymatic saccharification of (a) oil palm empty fruit bunch (OPEFB) and (b) sugarcane bagasse (SB) pretreated by liquid hot water (LHW) and 3% sulfuric acid at 121 °C for 1 hour with enzyme loading of 10, 20, and 30 FPU/ g substrate.

4. Conclusions
Biomass pretreated with H₂SO₄ produced higher reducing sugars than those pretreated by liquid hot water. Higher enzyme loading also resulted in higher reducing sugars. Reducing sugars obtained from enzymatic saccharification of OPEFB were higher than those obtained from SB. The highest total
reducing sugars (50.48 g/100 g biomass) was obtained from OPEFB pretreated with 3% H₂SO₄ at enzyme loading of 30 FPU per g biomass.

Acknowledgments
This study was supported by DIPA Project of the Research Center for Biomaterials, Indonesian Institute of Sciences in the fiscal year 2014 and also part of the Japan Science and Technology Agency (JST) – Japan International Collaboration Agency (JICA) for Science and Technology Research Partnership for Sustainable Development (SATREPS) Project Innovative Bioproduction Indonesia (IbioI): Integrated Bio-refinery Strategy to Promote Biomass Utilization using Super Microbes for Fuels and Chemicals Production (2013-2018).

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