Proceedings of the Pakistan Academy of Sciences:
B. Life and Environmental Sciences 57 (1): 43–50 (2020)

ISSN: 2518-4261 (print), ISSN 2518-427X (online)

Pakistan Academy of Sciences

Research Article

Mild Alkaline Pretreatment on Sugarcane Bagasse: Effects of Pretreatment Time and Lime to Dry Bagasse Ratio

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Abstract: Sugarcane waste is organic waste which has produced at about 8×10^6 tons annually in Indonesia. This lignocellulosic waste can be used as raw material for bioethanol manufacture through pretreatment, hydrolysis, and fermentation process. This study aims to evaluate system parameters of bagasse pretreatment using lime ($Ca(OH)_2$) in a batch system and to obtain the yield (g/g) of glucose produced from pretreated bagasse via enzymatic hydrolysis. The variables used in this study were pretreatment time and lime ratio to bagasse in the pretreatment step. It was then followed by a hydrolysis step to produce glucose. The fermentation was conducted to investigate how much ethanol can be produced at the best glucose yield. The results showed that pretreatment time gave a stronger effect on the glucose yield compared to lime ratio to bagasse. Longer pretreatment time showed the increased glucose yield in the hydrolysis of pretreated bagasse. However, after 180 minutes of pretreatment, glucose yield was decreased. Meanwhile, a higher ratio of lime to bagasse in the pretreatment process reduced the glucose yield. The best glucose yield (0.071 g/g) was obtained from the pretreatment process with a ratio of 0.5 g $Ca(OH)_2$ g^{-1} bagasse for 180 min; pH 5.0 and the ethanol content was (5.704 ± 0.15) g L^{-1}.

Keywords: Bagasse, Bioethanol, Enzymatic hydrolysis, Pretreatment.

1. INTRODUCTION

Most of the energy used comes from fossil fuels, which are non-renewable resources and become depleted. Sugarcane waste is a lignocellulosic material which is the potential to be converted into energy through a sugar-based fermentation process. Bagasse consists of three main components; those are hemicellulose. (25 %), cellulose (50 %), and lignin (25 %) [1]. Among the bagasse's content, lignin is the most inhibit component for enzyme performance in hydrolysis and reduces the sugar yield due to its complex structure. Therefore, bagasse has to be first processed before used as bioethanol feedstock.

In general, the pretreatment process for lignin removal in biomass is carried out chemically using acid reagents because it quickly produces desired results. However, the process usually requires corrosive reactants and high temperatures, thus, increasing the costs of material, waste treatment, and equipment maintenance. Chemical treatment will be more economical and environmental friendly when alkaline pretreatment is applied, since it leads to less solubilization of hemicellulose and less formation of inhibitor compounds, and does not require high temperature so the equipment maintenance costs will be reduced [2].

There are many reagents used in alkaline pretreatment to obtain good results which are also economic and environmentally friendly. The reagents which can be used are sodium hydroxide [3], sodium carbonate [4], potassium hydroxide [5], aqueous ammonia [6], and calcium hydroxide (lime) [7]. Sodium hydroxide is the best reagent in removing lignin, but it is unfriendly to the environment because it is difficult to recover and needs high energy for hydrolysis. Meanwhile, calcium hydroxide (lime) has been used as an alternative reagent because it needs low energy [2].

Received: September 2019; Accepted: March 2020
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Figure 1 shows the reaction of lime pretreatment on bagasse.

Several factors are playing an important role in bagasse pretreatments such as time, alkaline concentration, temperature, and lime ratio to bagasse. In this study, the use of lime for bagasse pretreatment is expected to meet environmental as well as economical aspects. The residue of lime pretreatment will be used for enzymatic hydrolysis which produces glucose and is used as raw material for bioethanol manufacture. The aims of this study are 1).to evaluate system parameters of bagasse pretreatment by using several variables of lime ratio to bagasse [(0.5, 1, 1.5, 2) g/g] and time pretreatment process [(60, 120, 180, and 240) min] 2). to determine the pH used for enzymatic hydrolysis and to estimate the yield of glucose produced in enzymatic hydrolysis from pretreated bagasse at those variables and certain pH. The ethanol content in the best glucose yield was also observed.

2. MATERIALS AND METHODS

2.1 Microorganism

Saccharomyces cerevisiae was obtained from dried yeast (Fermipan) bought from local suppliers. The organism was incubated in potato dextrose broth (PDB) at 28 °C for 20 h. The media was previously autoclaved at 121°C for 15 min.

2.2 Substrate

Bagasse is a substrate used in this study was obtained from PTPN-11 Surabaya. Bagasse was washed and dried in an oven (Memmert, Germany) at 60°C until its constant weight. The bagasse was reduced to get a homogeneous particle size (-40 mesh to 100 mesh) by using both grinder and screener (Retsch ASTM).

2.3 Pretreatment

Figure 2 shows the experimental scheme for the pretreatment process. In each batch mode, a 10 g dry bagasse was mixed with 150 mL $Ca(OH)_2$ solution with the ratio of $Ca(OH)_2$ to bagasse were 0.5, 1, 1.5, and 2 g/g. The stirrer was used at 200 rpm (1 rpm = 1/60 Hz) and the temperature was maintained in the range of 90 °C to 100 °C. Each experiment was conducted in 60, 120, 180, and 240 minutes. The sample was then washed with distilled water until neutral pH. The pretreated bagasse was dried at 60 °C to get its constant weight and used for enzymatic hydrolysis process.

2.4 Enzymatic Hydrolysis

A 2-gram bagasse which has been pretreated was used for enzymatic hydrolysis in a 50 mL working volume container. The enzyme used was a commercial liquid cellulase enzyme from *Trichoderma reesei* ATCC 26921 of 15 FPU g^{-1}. We used pretreated bagasse at ratio both 0.5 and 1 g/g and 60 minutes pretreatment to obtain the best pH from the highest amount of glucose. The pH variable used was 4.6; 5.0 and 6.0, which was controlled by adding 50 mL of citrate buffer solution [8]. The best pH was used for all pretreated bagasse in the hydrolysis process which was carried out in an incubator shaker for 72 h at 50°C and agitated at

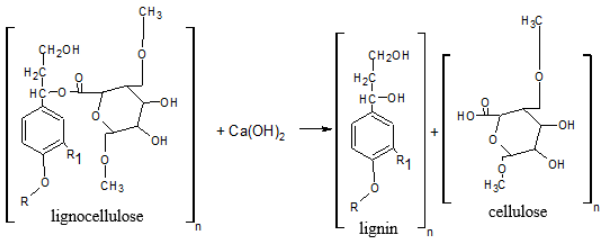

Fig. 1. The reaction of lime pretreatment on bagasse.

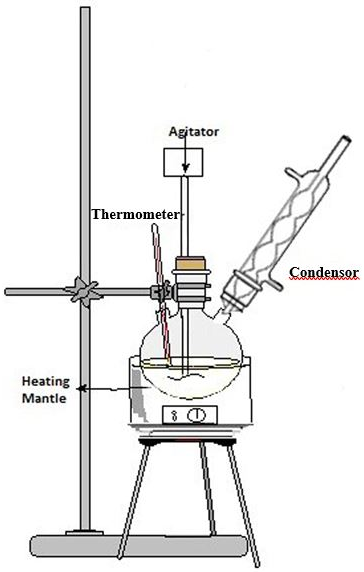

Fig. 2. Equipment scheme for lime pretreatment.

150 rpm. After 72 h, the enzyme activity was stopped by placing the sample at 100°C of the oil bath for 5 min. The solution was then filtered, and the filtrate was analyzed for its glucose content using the DNS method. The same amount of unpretreated bagasse was used in enzymatic hydrolysis as control.

2.5 Fermentation

Fermentation was performed in 25 mL working volume containers using 10 mL inoculum of yeast *Saccharomyces cerevisiae.* The substrate used for the fermentation was the hydrolysis sample from the bagasse pretreatment step at $Ca(OH)_2$ to bagasse ratio of 0.5 for 180 minutes which gave the best glucose yield in enzymatic hydrolysis. It was incubated at 30°C for 48 hours without agitation and analyzed for ethanol content for every 24 hours. Glucose for pro analytical grade from Merck with the same amount of glucose content in the hydrolysis sample was used as control.

2.6 Assay

Glucose content from enzymatic hydrolysis process was analyzed using the DNS method [9]. This DNS method was performed using a UV-VIS spectrophotometer (Hewlett Packard 8453) at a wavelength of 535 nm. Ethanol from the fermentation process was analyzed by Gas Chromatography (GC) (Hewlett Packard) using the HP Plot Q column at a speed of 15.2 mL min^{-1}. The degree of crystallinity in bagasse before and after the pretreatment was analyzed using X-Ray Diffraction (PhilipsXPert MPD).

3. RESULTS AND DISCUSSIONS

3.1 Effect of Lime Pretreatment to Substrate Structure

Alkaline pretreatment is a common method used to destruct lignin walls on the substrate; the alkaline agent used in this study was lime. The substrate used was bagasse that has passed the preparation stage, as depicted in Figure 3. Bagasse had an amorphous structure of lignin and a high degree of crystallinity so it can inhibit the effectiveness of the hydrolysis process. The decrease of lignin content and the increase of crystallinity degree in bagasse have led to higher cellulose content, which is expected to maximize the glucose yield from the subsequent hydrolysis process. The pretreatment of bagasse with lime to bagasse ratio broke down the amorphous structure of lignin and decreased the crystallinity degree of cellulose, as indicated in Figure 4.

X-Ray Diffraction (XRD) analysis was performed to determine the structure of sugarcane bagasse before and after alkaline pretreatment. Before alkaline treatment, cellulose is protected by lignin, which is shown as an amorphous structure (Figure 4) by XRD analysis. It can be seen that peak appears at $2\theta = 16°$ and 22° for bagasse prior pretreatment; the result is similar to XRD results in a previous study [10]. The degree of crystallinity is determined using the equation below:

$$\text{Degree of cristallinity} = A/(A+B) \quad (1)$$

A = crystalline area; B = amorphous area

Fig. 3. Sugarcane bagasse appearance before (left) and after (right) grinding.

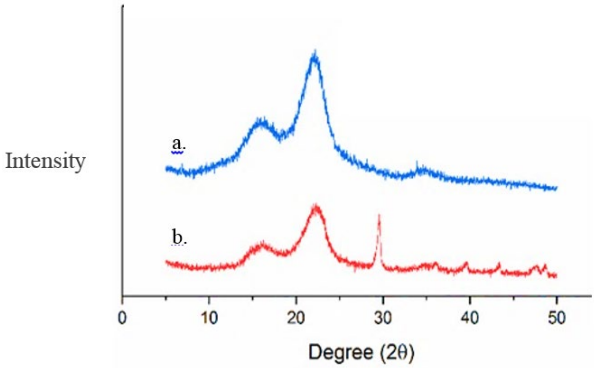

Fig. 4. XRD result of sugarcane bagasse before (a) and after lime pretreatment (b) for 0.5 g $Ca(OH)_2$ g^{-1} dry bagasse.

According to Eq (1) and XRD analysis, the degree of crystallinity of bagasse before and after the lime pretreatment (0.5 g $Ca(OH)_2$ g^{-1} dry bagasse) was found to be 31.9 % and 43.8 %, respectively. The increase in the degree of crystallinity after being subjected to lime pretreatment may be due to the dissolution of lignin and hemicellulose, which coated the cellulose of the bagasse.

3.2 Effect of pH on Enzymatic Hydrolysis of Bagasse

Pretreated bagasse samples at ratio lime to bagasse of 0.5 and 1 for 60 minutes pretreatment were enzymatically hydrolyzed at various pH. We chose only two variables which are the smaller ratio of lime to bagasse and the shortest period time of pretreatment to get the idea which pH was the best for the hydrolysis process as represented by the highest yield of glucose. Enzymatic hydrolysis was chosen to minimize unexpected side products and to apply a more environmental friendly process [9]. The enzyme used in this study was cellulase from *Trichoderma reesei* ATCC 26921, which is capable of converting cellulose into glucose (Figure 5).

The glucose yield increased by 4.9 % from pH 4.6 to pH 5 and decreased 4 % from pH 5 to pH 6 for the ratio of $Ca(OH)_2$/ bagasse of 0.5. The glucose yield increased by 3.19 % from pH 4.6 to pH 5 and decreased by 2.46 % from pH 5 to pH 6 for the ratio of $Ca(OH)_2$/ bagasse of 1.0. The unsuitable pH value can decrease ATP production, which is needed per mole of hydrolyzed cellulose so it can decrease the yield of glucose. The hydrolysis process at pH 5 gave the maximum yield of glucose as depicted in Figure 6. Hence, we used pH 5.0 for all pretreated bagasse in the hydrolysis process.

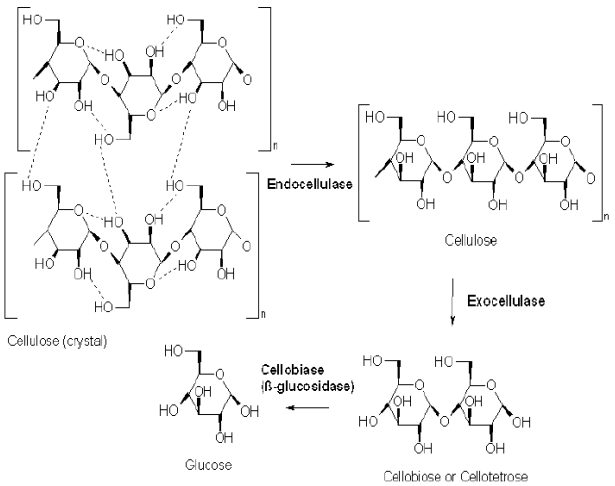

Fig. 5. Cellulose hydrolysis with cellulase enzyme.

3.3 Effect of Lime Concentration and Pretreatment Time on Enzymatic Hydrolysis of Bagasse

The glucose yield increased as a function of pretreatment time up to 180 min but decreased after 180 min as can be seen in Figure 7. A decrease in glucose yield after 240 min may be due to cellulose degradation to furfural, acetic acid, methanol, and other organic compounds due to prolonged pretreatment processing. The duration of pretreatment significantly influenced glucose formation for a ratio of 0.5 g and 1 g of calcium hydroxide/g dry bagasse, while at a ratio of 1.5 and 2 g $Ca(OH)_2$ g^{-1} dry bagasse did not show a significant increase in glucose yield. A higher ratio of lime to bagasse may more effective for removing lignin and also may cause more sugar degradation which may not be able to preserve most of the cellulose for further processing. The highest yield of glucose in each variation ratio of $Ca(OH)_2$ to bagasse was obtained at 180 min, so it implied that the pretreatment process is dominated by pretreatment time than ratio calcium hydroxide to bagasse. The glucose result from the control study was very small which was 0.00035±0.00002 g glucose/g bagasse.

Xu et al. [11] stated that the lime loading has a critical value of 0.1 g $Ca(OH)_2$ g^{-1} dry bagasse. The use of calcium hydroxide in the pretreatment

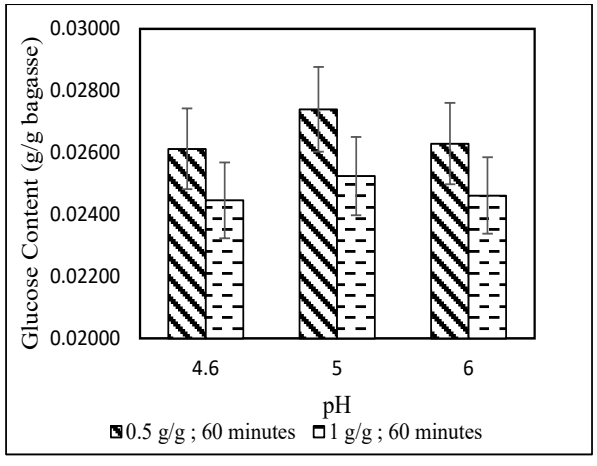

Fig. 6. Effect of pH on the yield of glucose from the hydrolysis process

process that exceeds the critical value will cause a decrease of cellulose content in bagasse. Unintentionally affected pretreatment at a higher ratio of lime concentration to dry bagasse on the reduction of cellulose concentration will lead to a reduction of glucose yield. The reduction of cellulose concentration is due to the dissolution of cellulose since the structure of cellulose is open during the pretreatment process [12]. The result from the study (Figure 7) shows that the highest glucose yield was obtained at 180 min with 0.5 g $Ca(OH)_2$ g^{-1} dry bagasse. A higher ratio of $Ca(OH)_2$/ dry bagasse shows lower glucose yield as can be seen as a percentage of glucose yield reduction (Table 1). Again, during the removal of hemicelluloses and/or lignin from the lignocellulose matrix can unavoidably cause cellulose to soluble, which leads to glucose yield reduction.

To achieve the maximum yield of glucose production, our study showed a shorter time of the pretreatment process compared to others [7, 13]. The pretreatment time is 224 and 6.7 times shorter compared to both previous results, respectively. However, the temperature used in this study is higher than in both the previous studies. A study conducted by Fajriutami et al. [3] using NaOH with a concentration of 1 % w/v at 121 °C gave a higher glucose yield compared to the glucose result in this study (Table 2). As $Ca(OH)_2$ was used as a pretreatment agent, the result from this study shows lower glucose yield compare to a previous study [13]. Sodium hydroxide is a better pretreatment agent compare to $Ca(OH)_2$ which gave higher glucose yield as shown by Fajriutami et al. [3], but NaOH is not environmental friendly compared to $Ca(OH)_2$ and also is more expensive. The comparison of our study to Rabello et.al [13] in the economic aspect lied on the time consumed and the temperature used for the treatment. The electricity used for the heating process at 90-100°C is not so big different from the process at 60°C. However, the length time processing for our study is 6.67 fold less than others [13] which will impact the cost of electricity used.

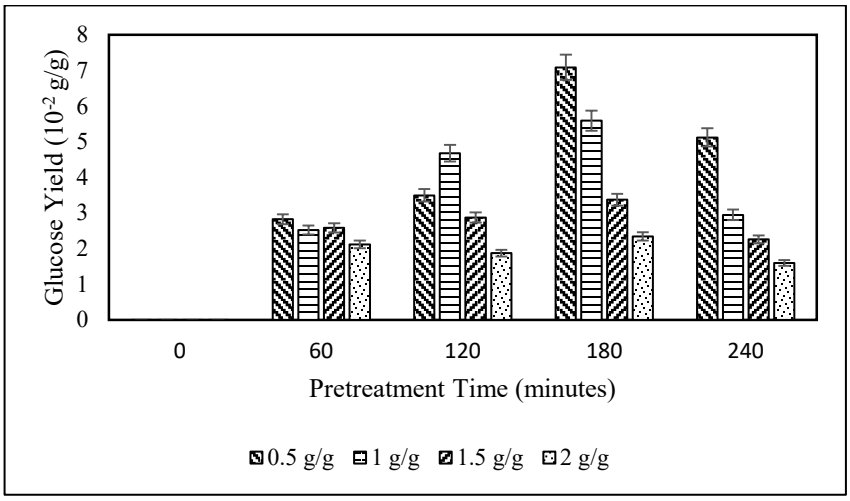

Fig. 7. Glucose yield on various ratio lime concentration to dry bagasse and pretreatment time.

Table 1. Glucose yield reduction at a various ratio of $Ca(OH)_2$/dry bagasse towards 0.5 g $Ca(OH)_2$/g dry bagasse

Ratio of $Ca(OH)_2$/dry bagasse	% Reduction in glucose yield
1	21.14
1.5	52.43
2	66.99

3.4 Ethanol Production in Fermentation Process

Fermentation was carried out to convert glucose to ethanol by *Saccharomyces cerevisiae.* Fermentation was performed based on the best result of the enzymatic bagasse hydrolyzed, which was a result of pretreatment at a ratio of 0.5 g $Ca(OH)_2$ g^{-1} dry bagasse and 180 min pretreatment time. In the fermentation process, the positive control solution was prepared by dissolving standard glucose with the same glucose content as in the sample solution (0.28 % b/v). This positive control solution was used as a reference to ensure that the fermentation procedure has been performed appropriately and to investigate the possible effects of impurities potentially present in the sample.

There is a difference between the ethanol yield from control and that from the sample (Figure 8), possibly due to the presence of inhibitors in the sample that can inhibit the fermentation process. Glucose used in the control solution has a high purity (pro analytic). Based on Li et al. [14], many inhibitors which can inhibit fermentation such as vanillin, phenol, furfural, syringaldehyde, formic acid, levilinic acid, and acetic acid. These inhibitors can be formed during the process of alkaline pretreatment and enzymatic hydrolysis.

The ethanol produced was higher (5.704 g/L) than the concentration of glucose used as a substrate, which is usually 50 % conversion based on stoichiometry, as the maximum theoretical yield of ethanol from glucose is 0.511g/g glucose. The presence of another carbon source (PDB) in the fermentation process was also calculated. The total glucose content in the substrate was approximate 9.68 g/L which comes from substrate and PDB (Potato Dextrose Broth). The PDB itself contains 20g/L dextrose. There is around 13.3 % deviation of the ethanol yield to a theoretical yield which needs further investigation. Table 3 summarizes the result of this study compared to the one done by Rabelo et al. [13].

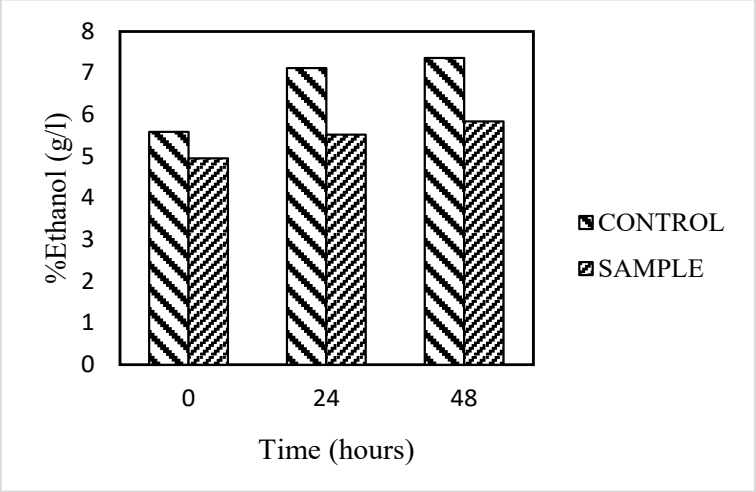

Fig. 8. Ethanol concentration for 48 hours fermentation

Table 2. Comparison of optimum conditions

Parameter	This research	Other research		
		Fajriutami et al. [3]	Kim et al. [7]	Rabelo et al. [14]
Substrate	Bagasse	Bagasse	Corncob	Bagasse
Pretreatment agent	$Ca(OH)_2$	NaOH	$Ca(OH)_2$	$Ca(OH)_2$
Concentration of pretreatment agent	0.5 g $Ca(OH)_2$/g dry bagasse	1 % b/v	0.05 g $Ca(OH)_2$/g biomass	0.25 g $Ca(OH)_2$/g dry bagasse
Pretreatment time	180 min	60 min	4 wk	1 200 min
Temperature	95 °C to 100 °C	121 °C	55 °C	60 °C
Yield of glucose	0.071 g/g dry bagasse	0.3397 g/g dry bagasse	0.93 g/g biomass	0.155 g/g dry bagasse

Table 3. Comparison of Fermentation Results

Parameter	This research	Other research by Rabelo et al. [14]
Substrate	Bagasse	Bagasse
Pretreatment agent	$Ca(OH)_2$	$Ca(OH)_2$
Concentration of pretreatment agent	0.5 g $Ca(OH)_2$ g^{-1} dry bagasse	0.25 g $Ca(OH)_2$ g^{-1} dry bagasse
Pretreatment time	180 min	1 200 min
Temperature	95 °C to 100 °C	60 °C
Enzyme used	15 FPU g^{-1} cellulase	3.5 FPU g^{-1} cellulase and 1 CBU/g β-glucosidase
Yield of glucose	0.071 g glucose g^{-1} dry bagasse	0.155 g glucose g^{-1} dry bagasse
Fermentation time	48 h	18 h
Yield of ethanol	5.8393 g L^{-1}	2.25 ± 0.1 g L^{-1}

The lower glucose yield resulted from our study may due to different conditions of pretreatment applied by Rabelo et al. [13] and also the availability of cellulose after pretreatment, while the lower ethanol yield reported by Rabelo et al. [13] might be due to differences in impurities contained in bagasse, fermentation conditions and also the availability of another carbon source.

4. CONCLUSIONS

Lime pretreatment time appeared to be a more dominant factor affecting the glucose yield compared to the ratio of lime to dry bagasse. The longer the time pretreatment process, the higher the yield of glucose produced from the subsequent hydrolysis process. The maximum pretreatment time is 180 min. A higher ratio of lime to the bagasse in the pretreatment process will lead to a reduction of glucose yield. The best glucose yield (0.071g/g) was obtained from the lime pretreated sample with a ratio of 0.5g $Ca(OH)_2$ g^{-1} dry bagasse, pH 5.0, and 180 min of pretreatment.

5. ACKNOWLEDGEMENTS

We thank Mr. Bagus Kurniawijaya from Bioprocess and Environmental Process Laboratory, UBAYA for the help with GC analysis.

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