MJFAS MALAYSIAN JOURNAL OF FUNDAMENTAL AND APPLIED SCIENCES
PRINT ISSN: 2289-5981 | ONLINE ISSN: 2289-599X

RESEARCH ARTICLE

Biohydrogen production from sugarcane bagasse pretreated with combined alkaline and ionic liquid [DMIM] DMP

Arief Widjaja [a,*], Silvya Yusnica Agnesti [a], Afan Hamzah [a], Hanny Frans Sangian [b]

[a] Department of Chemical Engineering, Institut Teknologi Sepuluh Nopember, Surabaya 60111, Indonesia
[b] Department of Physics, Sam Ratulangi University, Manado, 95115, Indonesia

* Corresponding author: arief_w@chem-eng.its.ac.id

Article history
Received 11 October 2018
Revised 20 December 2018
Accepted 26 February 2019
Published Online 15 October 2019

Abstract

Biohydrogen attracts many attentions since it has many advantages as source of energy. Biohydrogen from sugarcane bagasse offers many advantages from economic and environmental point of view. This work aimed to study the production of hydrogen from sugarcane bagasse through enzymatic hydrolysis and fermentation using *Enterobacter aerogenes*. Pretreatment with ionic liquid [DMIM]DMP was carried out prior to hydrolysis. It was found that process with ionic liquid was able to shift the cellulose structure from crystalline cellulose to more amorphous cellulose. Alkaline pretreatment followed by ionic liquid conducted for 20 min at 120°C gave the lowest crystallinity index. This condition also gave the highest total recovery of cellulose and hemicellulose, a condition that is very important for enzymatic hydrolysis to produce as much sugar as possible. Pretreatment condition was also found to give significant effect to the yield and type of monosaccharides produced from the hydrolysis process. Optimization of the pretreatment condition of the combined alkaline and ionic liquid [DMIM]DMP pretreatment was found to give significant effect to the ease of lignocellulosic substrate for its conversion to reducing sugar. The yield of hydrogen from the fermentation of the obtained sugar was 0.46 mole H_2 per mole of glucose consumed.

Keywords: Biohydrogen, sugarcane bagasse; ionic liquid; [DMIM]DMP pretreatment; *Enterobacter aerogenes*

INTRODUCTION

The world faces a problem of decreasing reserves of fossil fuels due to the ever-increasing need of energy. Fossil fuel also gives a negative effect to the environment as a result of its combustion that emits carbon dioxide, CO_2. For these reasons, researches on new sustainable energy sources that can substitute fossil fuel have been gaining more and more attention. Among several alternatives of biofuels such as biodiesel and bioethanol that have already been investigated, biohydrogen attracts many attentions since it has many advantages as an ideal source of energy. Burning hydrogen with air only produces water and no CO_2. Therefore, hydrogen will not contribute to global warming that resulted from the greenhouse effect, depletion of ozone or acid rain. Hydrogen also has the highest heating value of 122 MJ/kg compared to other fuels (Shanmugam *et al.*, 2003).

On the other hand, lignocellulosic wastes including sugarcane bagasse are abundantly presented in earth and can be utilized as the source of energy by enzymatic degradation followed by microbial fermentation (Saratale *et al.*, 2008).

The conversion of lignocellulose into biofuel is one of research interests by which successful enzymatic hydrolysis of cellulose to reducing sugar is much affected by pretreatment process (Muharja *et al.*, 2018). Delignification is one of important pretreatment steps since the existence of lignin restricts access of enzymes to breakdown cellulose and hemicellulose into sugar. Alkaline method is generally chosen since it produces minimum side products and gives lesser bad effect to the environment compared to acid method and it can run at a lower temperature (60-100 °C) (Rocha *et al.*, 2011).

The presence of hydrogen bonds in cellulose whether as intermolecular or intramolecular can hamper enzymatic degradation of cellulose due to the formation of crystalline cellulose (Azubuike *et al.*, 2011). Use of usual solvents is generally difficult to dissolve crystalline cellulose and hydrolyze it into reducing sugars (Arantes and Saddler, 2011). It has been the attention of many researches recently to develop specific solvents that are able to dissolve cellulose and reduce or eliminate the existence of crystalline cellulose (Arvela *et al.*, 2010).

Solvents can be classified into two groups, i.e. derivative and non-derivative solvents. Solvents that dissolve cellulose by converting it into derivatives of cellulose that are temporarily presented and classified as derivative solvent. Non-derivative solvent can dissolve cellulose by disrupting hydrogen bonds formation by which cellulose is not derived (Liebert, 2010). Some non-derivative solvents are used in cellulose pretreatment, for example concentrated phosphoric acid, N-methyl-morpholine-N-oxide and ionic liquids (Kuo and Lee, 2009).

Ionic liquids (ILs) are known to give a promising role as a non-derivative solvent, which is able to dissolve lignocellulosic materials (Bian *et al*., 2013). As a new type of solvent, ionic liquid attracts many researchers to dissolve cellulose due to its powerful characteristic; it can be recycled easily, has lower degree of toxicity, superior stability against heat and very low volatility (Chaumont and Wipff, 2007). It has been reported by Yang *et al.* (2010) that ionic liquid [DMIM]DMP has the highest effectivity in cellulose dissolution in comparison to five other alkyl-phosphate ionic liquids ([MEIM]DMP, [MAIM]DMP, [EMIM]DEP, [EEIM]DEP, [EAIM]DEP). This ionic liquid contributes an excellent performance in enzymatic saccharification process. [DMIM]DMP is also reported to be more powerful than [EMIM]DEP and [BMIM]DBP) due to its lowest viscosity, highest polarity and lowest ability for enzyme inhibition (Zhi *et al.*, 2012).

This research aimed to produce biohydrogen from sugarcane bagasse. Combination of alkaline and IL pretreatment using [DMIM]DMP was carried out prior to enzymatic hydrolysis. Microbial fermentation using *E. aerogenes* was conducted to convert sugars to biohydrogen. The effects of combination of NaOH and IL and other pretreatment conditions on sugar yield and types of fermentable sugar were studied and clarified.

MATERIALS AND METHODS

Materials

Sugarcane bagasse was obtained from one sugar factory under PTPN XI in Surabaya, Indonesia. It was crushed and sieved to around 60/80 mesh in size and dried in oven for 24 h at 60°C. Trimethyl phosphate (≥ 99.5%, 140.08 g/mol) and 1-methylimidazole (≥ 99%, 82.1 g/mol) were purchased from Sigma-Aldrich, St. Louise, MO, USA. All other chemicals were of commercial source.

Synthesis of ionic liquid [DMIM]DMP

Ionic liquid of 1,3-methylmethylimidazolium dimethyl phosphate, [DMIM]DMP was synthesized by the method previously described (Widjaja *et al.*, 2015; Sangian *et al.*, 2015a; Sangian *et al.*, 2015b). Synthesis of [DMIM][DMP] was conducted by reacting trialkyl phosphate and *N*-methylimidazole at 150°C for 15 h. After cooled to room temperature, the obtained ionic liquid was subjected to three times of washing by diethyl ether, and followed by stirring and heating for 24 h in an oil bath at 80°C so that all volatile residues could be eliminated. After that, IL was heated again for 24 h at 80°C prior to usage. The structure of IL was characterized and verified by 1H NMR (JNE-ECS-400MHz, JEOL, Japan). Figure 1 shows the reaction synthesis of [DMIM]DMP.

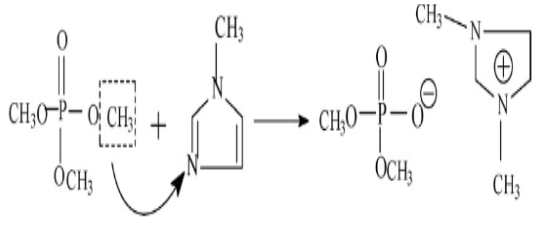

Figure 1 Synthesis of [DMIM]DMP from N-methylimidazole and trimethyl phosphate.

Alkaline pretreatment

Alkaline pretreatment was conducted by adding 1 L of 1% NaOH to 50 g sugarcane bagasse in a 2 L round bottom flask and heating by oil bath at 80°C for 16 h. After cooling and filtration, the obtained solid was washed with distilled water at temperature of 70°C until pH 7 was achieved before being dried at 60°C for 24 h in an oven.

Ionic liquid pretreatment

After alkaline pretreatment, 3.75 g of the sample was put in a 250 mL round bottom flask containing 100 ml ionic liquid [DMIM]DMP. Nitrogen gas was supplied to the solution to ensure atmospheric nitrogen so that the uptake of water from air could be avoided. The mixture was heated at 100-120°C for 10-900 min in an oil bath. As anti-solvent, ethanol was used to regenerate sugarcane bagasse from the solution (Widjaja *et al.,* 2015). Whatmann filter paper was then used to filter the solution to separate the precipitate, and then washed with distilled water and dried at 60°C for 48 h in an oven. The content of cellulose, hemicellulose and lignin was analyzed before preceding to enzymatic hydrolysis. To separate the ethanol, the used [DMIM][DMP] was subjected to distillation at a temperature of 100°C using oil bath and the ionic liquid could be reused again for another pretreatment.

Enzymatic hydrolysis

In 100 mL flask, 1.5 g sugarcane bagasse obtained under predetermined condition was added, followed by the addition of 45 mL citric buffer at pH 3 containing 27.9-unit cellulase and 27.9-unit xylanase. Hydrolysis was performed at 60°C for 12 h. At a certain time, interval, 1.5 mL sample was taken and centrifuged in order for the content of sugars in the supernatant to be measured.

Fermentation

Into 140 mL sugarcane bagasse, hydrolyzate 0.5% w/v yeast extract was added and the mixture was flushed with N_2 gas for 1 minute to remove oxygen from the medium. Sterilized 0.35 gr/L $FeSO_4.7H_2O$ by using UV for 15minute was added to the hydrolyzate. 10% volume from this was used for acclimatization into which *E. aerogenes* NBRC 13534 that has been cultivated for 1 day in PDA medium was inoculated and the growth of the bacterium was conducted for 14-16 h until the concentration of cells was more than 10 million cells/mL. Then this preculture solution was mixed with 90% volume of the hydrolyzate for biohydrogen production study. Since the bacterium used was a strict anaerobic, both the preculture and the fermentation were conducted at nitrogen atmosphere with careful isolation to avoid contact with air. The fermentation was performed at temperature of 30°C by shaking at 100 rpm. The gas produced was collected in an aluminum gas collection bag connected to the bottle through a poly (vinyl chloride) tube. 3 ml sample was taken every 6 hour for glucose assay and number of cells. Hydrogen concentration was analyzed using gas chromatography.

Method of analysis

Cellulose, hemicellulose and lignin concentrations in sugarcane bagasse were measured by the method reported by Datta (1981). Sugar assay was conducted using DNS method (Miller, 1959) with glucose as standard by following the absorbance using spectrophotometer at 540 nm.

The crystalinity index was calculated from XRD pattern by comparing surface area of crystalline to that of total crystalline and amorphous (Poletto, 2012).

RESULT AND DISCUSSION

Effect of particle size during alkaline pretreatment

The effect of particle size of sugarcane bagasse during alkaline pretreatment is shown in Table 1. This table shows that pretreatment with 1% NaOH resulted in a significant decrease of lignin content, i.e. around 60% of decrease. However, the figure also shows that smaller particle size gave practically no difference in the degradation of lignin. Larger particle could also minimize the loss of solid residue. This loss may due to dissolution of solid in the alkaline solution and loss in filter paper during filtration procedure. The smaller loss of the solid residue will therefore lead to higher recovery of cellulose and hemicellulose required to produce fermentable sugar. Smaller particle size of < 60 mesh was then used for further experiments.

XRD pattern of sugarcane bagasse

Figure 2 shows the XRD pattern of substrates pretreated with NaOH and ionic liquid. The untreated sample and the sample pretreated with alkaline alone showed a typical cellulose diffraction pattern with a high structure and peaks at the angles (16.00º, 22.00º and 35.00º), which correlated to reflector planes of (101), (002) and (040), respectively. This was comparable to previous results (Park *et al.*, 2010; Zhao *et al.*, 2012; Sun *et al.*, 2009). As can be seen from the figure, pretreatment with ionic liquid resulted in a peak shift due to expansion of the lattice (Zhu *et al.*, 2012). It also can be seen from the graph that substrate pretreated with IL encountered a change in its symmetry as obviously observed by a slight movement to the right of the angle 2θ around 0.50º to 1.00º. Pretreatment with NaOH alone gave no peak shift to prove that the shift of peak only occurred when IL was applied.

Table 1 Effect of particle size during alkaline pretreatment of sugarcane bagasse.

Particle size	Content in solid residue (%)			Loss of solid residue	Recovery of C and H (%)
	C	H	L		
Untreated	32.8%	26.3%	24.8%	-	-
1% NaOH treated of < 60 mesh bagasse	67.1%	17.5%	9.8%	52 %	69%
1% NaOH treated of 100/120 mesh bagasse	63.4%	18.1%	9.8%	64 %	50%

C, H and L represent cellulose, hemicellulose and lignin, respectively.

The figure also shows the crystallinities of various substrates used in the present experiment. After pretreated with alkaline alone, it can be seen from the figure that sugarcane bagasse crystallinity was increased in comparison to the untreated one due to the removal of amorphous hemicellulose and lignin. Lignin and hemicellulose were dissolved by alkaline solution, leading to the exposure of the cellulose (Sghaier *et al.*, 2012). The untreated sugarcane bagasse has the lowest crystallinity index since it contained with the highest lignin in the outer space of cellulose structure.

After pretreated with ionic liquid [DMIM]DMP for 10 min and 120°C, the Cr.I was decreased as compared to pretreatment using NaOH alone, and the value was further improved (smaller) when pretreatment time was continued for 20 min. However, when the time of ionic liquid dissolution was continued to 1 h and 15 h, the Cr.I was increased back to higher value. The results revealed that there was an optimum ionic liquid pretreatment time and temperature that gave the lowest index of crystallinity. The lowest crystallinity index was obtained from the sample that pretreated by alkaline and followed by ionic liquid for 20 min at 120 °C. The reason for this low index is that crystalline cellulose in sugarcane bagasse is changed into more amorphous one compared to the substrate pretreated with NaOH only. In the enzymatic process, this amorphous cellulose can be hydrolyzed easier by enzyme.

Effect of alkaline and ionic liquid pretreatment condition on the recovery of cellulose and hemicellulose

The mechanism of dissolution of cellulose in ionic liquid solution is shown in Figure 3, which adapted from Feng and Chen (2008). Cellulose can dissolve perfectly in ionic liquid solution due to the formation of hydrogen bond between anion of ionic liquid and -OH in cellulose (Liu *et al.*, 2012).

During the dissolution of the substrate in ionic liquid solution, all components including cellulose, hemicellulose and lignin are dissolved. After adding anti-solvent ethanol in the regeneration step, the hydrogen bond between cellulose and ionic liquid is broken down and the liquid cellulose can then be regenerated back to form a solid cellulose. The present work studied the effect of alkaline and ionic liquid pretreatment condition on the recovery of cellulose and hemicellulose. Pretreatment was conducted for 10 min to 900 min whereas the temperature of the process was set at 100 and 120°C. Higher temperature than 120ºC was once conducted but it gave bad results (data not shown).

Table 2 shows the effect of time and temperature during ionic liquid pretreatment on the cellulose and hemicellulose recovery. It can be seen from this table that the best recovery of cellulose and hemicellulose was obtained when pretreatment was performed using alkaline and followed by ionic liquid for not more than 20 min at 100 or 200ºC. Total recovery of cellulose and hemicellulose is very important since in the hydrolysis process, these two components will be converted to fermentable sugar. The table also shows that the smallest loss of solid was obtained by NaOH pretreatment, followed by ionic liquid at 20 min and 120ºC.

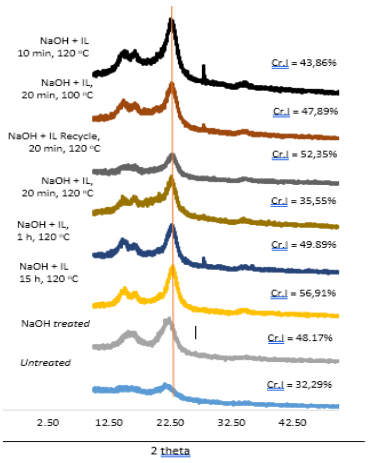

Figure 2 XRD pattern of various solid residues used in the present experiment.

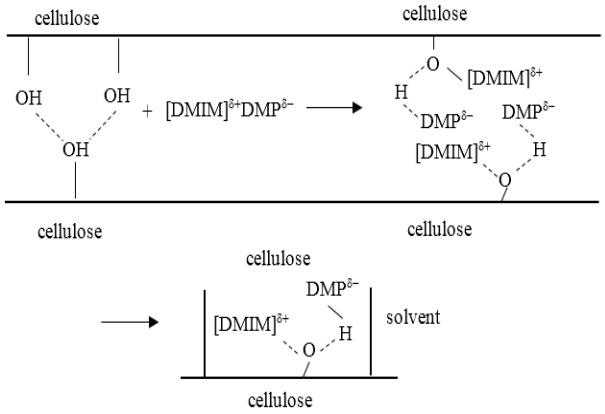

Figure 3 Mechanism of dissolution of cellulose in ionic liquid [DMIM][DMP].

The result of the best total recovery of cellulose and hemicellulose is given in Table 2 and it coincides with the result of the lowest crystallinity index shown in Figure 2. The best ionic liquid pretreatment time and temperature also resulted in a good retention of hemicellulose.

Table 2 Effect of pretreatment condition to the recovery of cellulose and hemicellulose.

Pretreatment	Content (%)			A*)	B*)
	Cellulose	Hemi	Lignin		
Untreated	32.87%	26.30%	24.81%	-	-
NaOH treated	67.19%	17.53%	9.84%	-	-
NaOH+IL 10 min 120°C	65.23%	12.64%	7.85%	6%	90%
NaOH+IL 20 min 120°C	58.49%	18.02%	6.34%	2%	92%
NaOH+IL 1 h 120°C	55.39%	8.67%	6.10%	18%	65%
NaOH+IL 15 h 120°C	60.66%	13.45%	7.80%	18%	75%
NaOH+IL 20 min 100°C	64.15%	14.83%	9.14%	3%	93%
NaOH+IL Recycle 20 min 120°C	59.11%	6.53%	9.51%	5%	76%

A*) Loss of solid after pretreatment
B*) Recovery of cellulose and hemicellulose

Production of sugar from sugarcane bagasse

The effects of the condition of pretreatment on the concentration and yield of sugar are shown in Figure 4. It can be shown from the figure that the highest reducing sugar was obtained from sugarcane bagasse pretreated with NaOH, followed by [DMIM]DMP that conducted for 20 min at 100°C or 120°C. The maximum yield of 0.56 g sugar per g of cellulose and hemicelluloses was attained at temperature of 120°C. This result showed that pretreatment time using ionic liquid [DMIM]DMP was 20 min in order to give optimum yield of sugar. At this condition, cellulose can dissolve and regenerate well without too much loss of hemicellulose. The results of the experiment were in good agreement with the data in Table 2 and Figure 2. It can be seen that the performance of reuse ionic liquid [DMIM]DMP was similar to the new one. Use of individual [DMIM]DMP alone without alkaline pretreatment gave worse result than using alkaline pretreatment prior to ionic liquid (data not shown). This result showed that the removal of lignin by alkaline pretreatment was required to provide effective dissolution of cellulose and hemicellulose.

Table 3 shows the results of comparison between total reducing sugar measured using DNS method and its corresponding individual monosaccharide concentration measured by HPLC. As seen from the table, different pretreatment conditions will give different compositions of monosaccharides. Both glucose and xylose seem to be the major monosaccharides produced after enzymatic hydrolysis of sugarcane bagasse. It can be seen from the table that the total sugar concentration measured by HPLC was significantly less than the total reducing sugar measured by DNS. One possibility is that HPLC can not measure all sugars in the form of monosaccharides that contained in the hydrolysate. This will require further work to clarify this problem. However, it can be seen from the table that pretreatment with IL gave better yield of sugar compared to that without IL pretreatment.

The results presented in this work revealed that utilization of alkaline pretreatment followed by ionic liquid [DMIM]DMP was a promising method to hydrolyze sugarcane bagasse into reducing sugar. The most disadvantageous factor that hampers the application of this method is the expensive price of ionic liquid. The cost of ionic liquid contributes around 93% of the total production cost of converting biomass to sugars as reported by Sen *et all*. (2012). The MSP (minimum selling price) of fermented sugar, at break event point, is significantly higher than the current market price of sugars. It is obviously shown that this method still can't compete with fossil fuels. Therefore, process configuration that required lower ionic liquid consumption should be investigated. Lower ionic liquid can be investigated by lowering ionic liquid concentration. Focus of this work was to investigate the effect of new and recycle ionic liquid [DMIM]DMP in combination with NaOH pretreatment and to find the right condition of the pretreatment. Therefore, the effect of ionic liquid concentration will be conducted in the next work. As reported by Fu and Mazza (2011) who employed ionic liquid [EMIM]Ac with wheat straw as the substrate, fermentable sugars recovery has an optimum processing condition at temperature of 158°C for 3.6 h under ionic liquid concentration of 49.5% (w/w). This indicated that lowering concentration of ionic liquid was allowed.

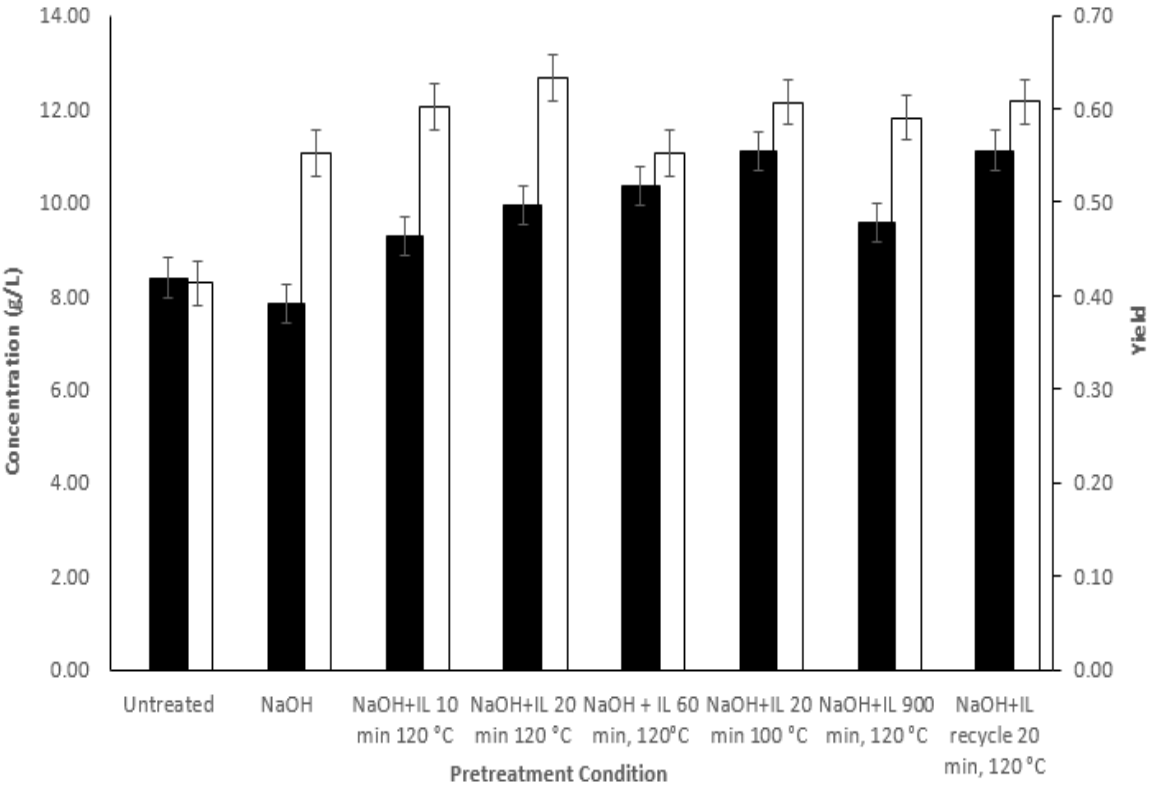

Figure 4 Effect of pretreatment condition on sugar concentration (□) and yield (■).

Table 3 Comparison of total reducing sugar and its corresponding individual monosaccharide.

Condition	HPLC Analysis					Total reducing sugar
	Glu	Xyl	Arab	Man	Total	
	(g/L)	(g/L)	(g/L)	(g/L)	(g/L)	(g/L)
Untreated	1.38	0.02	1.26	-	2.65	8.27
NaOH treated	0.13	0.49	2.13	-	2.75	11.04
NaOH + IL 10 min 120°C	1.63	0.42	2.03	-	4.07	12.04
NaOH + IL 20 min 120°C	-	0.09	0.84	-	0.93	12.66
NaOH + IL 1 h 120°C	0.31	0.42	-	0.07	0.80	12.39
NaOH + IL 15 h 120°C	2.16	-	-	-	2.16	11.81
NaOH + IL 20 min 100°C	2.64	-	-	-	2.64	12.14
NaOH + IL Recycle 20 min 120°C	0.29	1.54	-	-	1.83	6.39

Glu, Xyl, Arab and Man represent for glucose, xylose, arabinose and mannose, respectively, as measured by HPLC. Total reducing sugar was measured by DNS method

Fermentation of sugarcane bagasse

The results of fermentation of sugar obtained from enzymatic hydrolysis are shown in Figure 5 and 6. It can be seen from Figure 5 that sugar was consumed by the cells of *E. aerogenes* for their growth. The cell growth rate was high until 30 h and began to enter stationary phase afterward, even though the sugar consumption was still continuing. The sugar consumption rate began to stop at 42 h. It may be assumed from figure that after 42 h, the consumption of sugar is not resulted from cell activation to do fermentation process.

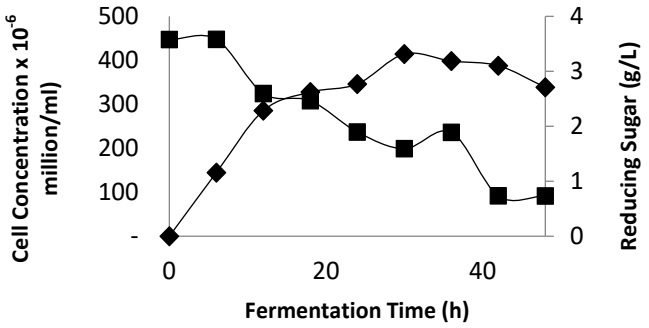

Figure 5 Time course of cell (◆) and reducing sugar (■) concentration during fermentation of sugarcane bagasse.

Figure 6 shows the time course of the accumulation of the product hydrogen during fermentation process. It can be seen from the figure that the production of hydrogen stopped at 30 h. The result coincided with the end of cell growth at 30 h in Figure 5. Argun *et al.* (2008) reported that the formation of by-product in the fermentation process such as the production of some acids was one of the factors that inhibited the activation of cells. The reason for the decreasing of sugar concentration after the cells was stopped to grow should be clarified in a future work. The yield of hydrogen was 0.46 mole H_2 per mole of reducing sugar consumed.

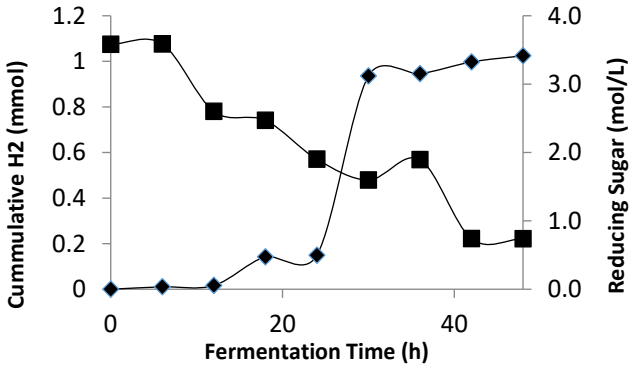

Figure 6 Time course of cumulative H_2 (◆) and reducing sugar (■) concentration during fermentation of sugarcane bagasse.

CONCLUSION

Combined pretreatment of alkaline followed by ionic liquid [DMIM] DMP was able to shift the cellulose structure from crystalline cellulose to more amorphous cellulose. Ionic liquid pretreatment conducted for 20 min at 120ºC gave the lowest crystallinity index. This condition also gave the highest total recovery of cellulose and hemicellulose, a condition that is very important for enzymatic hydrolysis to produce as much sugar as possible. Pretreatment condition was also found to give significant effect on the yield and type of monosaccharides produced from the hydrolysis process. Optimization of the pretreatment condition of the combined alkaline and ionic liquid [DMIM]DMP pretreatment was found to give significant effect on the ease of lignocellulosic substrate to be converted into sugar for biohydrogen production. The sugar produced through the optimum condition of the combined alkaline and ionic liquid pretreatment followed by enzymatic hydrolysis gave hydrogen yield of 0.46 mole H_2 per mole of glucose consumed during fermentation process.

ACKNOWLEDGMENTS

This research was funded by Ministry of Research and Technology and Higher Education of Indonesia. The authors would like to express sincere thanks for the support.

REFERENCES

Arantes, V., Saddler, J. N. (2011). Cellulose accessibility limits the effectiveness of minimum cellulase loading on the efficient hydrolysis of pretreated lignocellulosic substrates. *Biotechnology for Biofuels, 4*, 3.

Argun, H., Kargi, F., Kapdan, I. K., Oztekin, R. (2008). Batch dark fermentation of powdered wheat starch to hydrogen gas: Effects of the initial substrate and biomass concentrations. *International Journal of Hydrogen Energy, 33*(21), 6009 - 6115.

Mäki-Arvela, P., Anugwom, I., Virtanen, P., Sjöholm, R., Mikkola, J. P. (2010). Dissolution of lignocellulosic materials and its constituents using ionic liquids-A review. *Industrial Crops and Products,* 32(3), 175–201.

Azubuike, C. P., Rodríguez, H., Okhamafe, A. O., Rogers, R. D. (2011). Physicochemical properties of maize cob cellulose powders reconstituted from ionic liquid solution. *Cellulose, 19*(2), 425-433.

Bian, J., Peng, F., Peng, X. P., Xiao, X., Peng, P., Xu, F., Sun, R. C. (2013). Effect of [Emim]Ac pretreatment on the structure and enzymatic hydrolysis of sugarcane bagasse cellulose. *Carbohydrate Polymer, 100*, 211-217.

Chaumont, A., Wipff, G. (2007). Solvation of big spherical solutes in room temperature ionic liquids and at their aqueous interface: A molecular dynamics simulation study. *Journal of Molecular Liquids, 131–132*, 36–47.

Datta, R. (1981). Acidogenic fermentation of lignocellulose-acid yield and conversion of components. *Biotechnology and Bioengineering, 23*, 2167-2170.

Fu, D., Mazza, G. (2011). Optimization of processing conditions for the pretreatment of wheat straw using aqueous ionic liquid. *Bioresource Technology, 102*(17), 8003-8010.

Kuo, C. H., Lee, C. K. (2009). Enhancement of enzymatic saccharification of cellulose by cellulose dissolution pretreatments. *Carbohydrate Polymers*, *77*(1), 41–46.

Liebert, T. (2010). Cellulose solvents- remarkable history, bright future. In T. F. Liebert, T. J. Heinze, & K. J. Edgar (Eds.), *Cellulose solvents: For analysis, shaping and chemical modification* (pp. 3–54). New York: Oxford University Press.

Liu, W., Hou, Y., Wu, W., Ren, S., Wang, W. (2012). Complete conversion of cellulose to water soluble substance by pretreatment with ionic liquids. *Korean Journal of Chemical Engineering*, *29*(10), 1403-1408.

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry, 31*(3), 426-428.

Muharja, M, Junianti, F, Ranggina, D., Nurtono, T., Widjaja, A. (2018). An integrated green process: Subcritical water, enzymatic hydrolysis, and fermentation, for biohydrogen production from coconut husk. *Bioresource Technology, 249*, 268–275.

Park, S., Baker, J. O., Himmel, M. E., Parilla, P. A., Johnson, D. K. (2010). Cellulose crystallinity index: Measurement techniques and their impact on interpreting cellulase performance. *Biotechnology for Biofuels, 3*(10), 1-10.

Poletto, M., Ademir J. Z., Maria, M. C. F., Ruth M C. S. (2012). Thermal decomposition of wood components and cellulose crystallite size. *Bioresource Technology*, 109, 148-153.

George, J. M. R., Martin, C., da Silva, V. F. N., Gómez, E. O., Goncalves, A. R. (2011). Mass balance of pilot-scale pretreatment of sugarcane bagasse by steam explosion followed by alkaline delignification. *Bioresource Technology, 111*, 447-452.

Sangian, H. F., Kristian, J., Rahma, S., Agnesty, S. Y., Gunawan, S., Widjaja, A. (2015a). Comparative study of the preparation of reducing sugars hydrolyzed from high-lignin lignocellulose pretreated with ionic liquid, alkaline solution and their combination. *Journal of Engineering and Technological Sciences, 47*(2), 137-148.

Sangian, H. F., Kristian, J., Rahma, S., Dewi, H. K., Puspasari, D. A., Agnesty, S. Y., Gunawan, S., Widjaja, A. (2015b). Preparation of reducing sugar hydrolyzed from high-lignin coconut coir dust pretreated by the recycled ionic liquid [mmim][dmp] and combination with alkaline. *Bulletin of Chemical Reaction Engineering & Catalysis, 10*(1), 8-22.

Saratale, G. D., Chen, S. D., Lo, Y. C., Saratale, R. G., Chang, J. S. (2008). Outlook of biohydrogen production from lignocellulosic feedstock using dark fermentation. A review. *Journal of Scientific and Industrial Research*, *67*(11), 962-978.

Sen, S. M., Binder, J. B., Raines, R. T., Maravelias, C. T. (2012). Conversion of biomass to sugars via ionic liquid hydrolysis: process synthesis and economic evaluation. *Biofuels, Bioproducts & Biorefining, 6*(4), 444–452.

Sghaier, A. E. O. B., Chaabouni, Y., Msahli, S., Sakli, F. (2012). Morphological and crystalline characterization of NaOH and NaOCl treated *Agave americana L.* fiber. *Industrial Crops and Products, 36*(1), 257–266.

Shanmugam, S., Hari, A., Ulaganathan, P., Yang, F., Krishnaswamy, S., Wu, Y.R. (2018). Potential of biohydrogen generation using the delignified lignocellulosic biomass by a newly identified thermostable laccase from *Trichoderma asperellum* strain BPLMBT1. *International Journal of Hydrogen Energy*, 43, 3618–3628.

Sun, Y., Zhuang, J., Lin, L., Ouyang, P. (2009). Clean conversion of cellulose into fermentable glucose. *Biotechnology Advances, 27*(5), 625-632.

Widjaja, A., Agnesty, S. Y., Sangian, H. F., Gunawan, S. (2015). Application of ionic liquid [DMIM]DMP pretreatment in the hydrolysis of sugarcane bagasse for biofuel production. *Bulletin of Chemical Reaction Engineering & Catalysis, 10*(1), 70-77.

Yang, F., Li, L., Li, Q., Tan, W., Liu, W., Xian, M. (2010). Enhancement of enzymatic in situ saccharification of cellulose in aqueous-ionic liquid media by ultrasonic intensification. *Carbohydrate Polymers, 81*(2), 311-316.

Zhao, D., Li, H., Zhang, J., Fua, L., Liu, M., Fua, J., Ren, P. (2012). Dissolution of cellulose in phosphate-based ionic liquids. *Carbohydrate Polymer, 87*(2), 1490-1494.

Zhi, S., Liu, Y., Yu, X., Wang, X., Lu, X. (2012). Enzymatic hydrolysis of cellulose after pretreated by ionic liquids: focus on one-spot process. *Energy Procedia, 14*, 1741-1747.

Zhu, Z., Zhu, M., Wu, Z. (2012). Pretreatment of sugarcane bagasse with NH4OH–H2O2 and ionic liquid for efficient hydrolysis and bioethanol production. *Bioresource Technology, 119*, 199–207.