Research Article

Preparation of Reducing Sugar Hydrolyzed from High-Lignin Coconut Coir Dust Pretreated by the Recycled Ionic Liquid [mmim][dmp] and Combination with Alkaline

Hanny F. Sangian, Junaidi Kristian, Sukmawati Rahma, Hellen Kartika Dewi, Debra Arlin Puspasari, Silvya Yusnica Agnesty, Setiyo Gunawan, Arief Widjaja*

Department of Chemical Engineering, Faculty of Industrial Technology, Sepuluh Nopember Institute of Technology, Keputih Sukolilo, Surabaya 60111, Indonesia

Abstract

This study aims to produce reducing sugar hydrolyzed from substrate, coconut coir dust pretreated by recycled ionic liquid and its combination with alkaline. The ^{1}H NMR and FTIR were performed to verify the synthesized ionic liquid methylmethylimidazolium dimethyl phosphate ([mmim][dmp]). The structure of pretreated substrates was analyzed by XRD measurement. The used ionic liquid was recycled twice to re-employ for substrate pretreatment. The treated- and untreated-coconut coir dust were hydrolyzed into sugars using pure cellulase. The reaction, which called an enzymatic hydrolysis, was conducted at 60 °C, pH 3, for 48 h. The yields of sugar hydrolyzed from fresh IL-pretreated, 1R*IL-pretreated and 2R*IL-pretreated substrates were of 0.19, 0.15 and 0.15 g sugar / g cellulose+hemicellulose, respectively. Pretreatment with NaOH or the combination of NaOH+IL resulted in yields of reducing sugars of 0.25, 0.28 g/g, respectively. When alkaline combined with the recycled ionic liquids, NaOH+1R*IL, NaOH+2R*IL in the pretreatment, the yields of sugar were relatively similar to those obtained using alkaline followed by fresh ionic liquid. If the mixture enzymes, cellulase+xylanase, used to liberate sugars from fresh IL-pretreated, or recycled IL-pretreated substrates, the amount of sugar (concentration or yield) increased slightly compared to that employing a single cellulase. These findings showed that recycled IL pretreatment of the high-lignin lignocellulose, coconut coir dust, is a new prospect for the economical manufacture of fermentable sugars and biofuel in the coming years. © 2015 BCREC UNDIP. All rights reserved

How to Cite: Sangian, H.F., Kristian, J., Rahma, S., Dewi, H., Puspasari, D., Agnesty, S., Gunawan, S., Widjaja, A. (2015). 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. (doi:10.9767/bcrec.10.1.7058.8-22)

Permalink/DOI: http://dx.doi.org/10.9767/bcrec.10.1.7058.8-22

1. Introduction

Coconut coir dust, as lignocellulosic material, is composed of cellulose, hemicellulose, lignin and ash, whose the chemical compositions are different in each biomass. The cellulosic chain has a strong connection called a β (1→4) glycosidic bond, and an H bond at the hydroxyl OH functions as a protector for sugar monomers [1]. Biomass pretreatments, including lignin dissolution with chemical and physical

* Corresponding Author.
 E-mail: arief_w@chem-eng.its.ac.id
 Telp: Telp: +62-31-5946240, Fax: +62-31-5999282

techniques, have been conducted to change the crystalline orientation of cellulose, thus improving the yield of fermentable sugar in enzymatic hydrolysis [2-4]. Chemical methods have been employed to carry out pretreatment on grape stalks to regain both hemicellulose and cellulose [5]. However, the wastewater, which contained chemical substances was difficult to be separated. The sub- and supercritical methods have disadvantages as follows: production cost is still very high, the technology is difficult to scale up to an industrial scale because of safety consideration [6].

Ionic liquid (IL) as an agent for cellulosic dissolution has been applied due to its superiority compared to old solvents. The advantages are their low vapor pressure, low melting point, adjustable acidity, ability to be recycled and environmentally friendly properties [7, 8]. Waste of sugar industry called sugarcane bagasse has been successfully converted into fermentable sugars and bioethanol by introducing IL 1-allyl-3-methylimidazolium chloride [[amim] Cl] technique. This ionic liquid successfully dissolved sugarcane bagasse (SCB), and its crystal index decreased by 42%, so the enzymatic hydrolysis efficiency was improved significantly. The ionic liquid pretreatment did not adversely predispose the fermenting process [9]. Liquefying the biomass into ionic liquid induces the change of the lattice orientation from high- to low-crystalline structures that are easily hydrolyzed into reducing sugar and then yeasted into biofuel [10-13].

However, the price of reactants to synthesize ionic liquid, or manufactured ionic liquid, is still expensive so that it is a major hindrance of ionic liquid pretreatment [14]. The option to reduce the production cost of reducing sugar is to reuse of recycled ionic liquid for biomass pretreatment.

Ionic liquid 1-ethyl-3-methylimidazolium diethylphosphate [emim][dep] was successfully recycled after pretreating a pure cellulose [14]. The SCB has been hydrolyzed into reducing sugar using recycled ionic liquid [amim][Cl] and it was found surprisingly that there was no significant difference of sugar yield hydrolyzed from substrates pretreated by new ionic liquid and recycled ionic liquid [9,15]. The study showed sugarcane bagasse could be converted easily into sugars employing a recycled ionic liquid 1-ethyl-3-methylimidazolium acetate [emim][oac] pretreatment. The yield of reducing sugar decreased as the number of pretreatment recycles increased but the decrease was not significant [15].

This work followed some steps as follows:

manufacture of ionic liquid; substrates pretreatment by recycled ionic liquid, alkaline followed by recycled ionic liquid and enzymatic hydrolysis of pretreated coconut coir dust into fermentable sugars. The IL [mmim][dmp] was synthesized by the reaction of 3-methyl phosphate, [tmp], and 1-methylimidazole, [mim], at 150 °C.

The NMR (*nuclear magnetic resonance*) and FTIR (*Fourier transform infra-red*) analysis were used to verify the ionic liquid structure, followed by the pretreatment of coconut coir dust using the ionic liquid and recycled ionic liquid. The crystalline orientations of all substrates, non-pretreated and pretreated lignocelluloses, were characterized by XRD.

Finally, the pretreated and non-pretreated coconut dust lignocelluloses were hydrolyzed into reducing sugars by pure cellulase. The total reducing sugars were measured by the DNS (*dinitrosalicylic acid)* method, and the sugar fractions (glucose, xylose and galactose) were analyzed by HPLC (high-performance liquid chromatography). The yields or concentrations of reducing sugar hydrolyzed from substrates, which pretreated by new ionic liquid, recycled ionic liquid, alkaline and their combination were compared.

2. Materials and Methods

2.1. Material Preparation

Coconut coir dust was obtained costless from a coir factory in South Minahasa, North Sulawesi, Indonesia. Trimethyl phosphate ($\geq$ 99.5%, 140.08 g/mol) and 1-methylimidazole ($\geq$ 99%, 82.1 g/mol) were purchased from *Sigma-Aldrich, St. Louise MO USA*. The manufacture of the ionic liquid, *1,3-methylmethylimidazolium dimethyl phosphate*, followed procedure which was based on previously reported techniques [16-18].

The steps of synthesizing IL, are: reactants, [tmp] and (mim) were weighed to form the identical molar substance and the reactor was purged by N_2 gas for 5 min to remove water vapor. The (tmp) and (mim) were reacted inside round bottom flask at normal conditions, one bar, temperature at 150 °C for 15 h under vigorous stirring. The IL synthesis was exothermic, second order reaction and was simplified as follows:

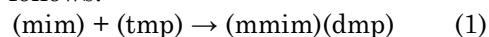$$(mim) + (tmp) \rightarrow (mmim)(dmp) \qquad (1)$$

Table 1 shows the amount of reactants and IL product in an identical molar reaction, whose it is exothermic characterization, second order reaction.

The verification of IL structural characterization was performed by ^{1}H NMR (JNE/ECS/400MHz-JEOL, Japan). The procedure of this work is shown in Figure 1. There were two ways in this pretreatment until fermentable sugars as follows: the substrate was treated firstly by alkaline for delignification and the substrate was directly treated by fresh or recycled ionic liquid.

Six grams of the lignocellulosic material, 120 mesh, was mixed in 250 ml one percent alkaline at 80 °C for 16 h, and then the solid was washed with hot water. The next step was pretreatment of lignocellulose using the new ionic liquid and recycled ionic liquid as developed in a previous work [14]. One hundred milliliters of the new ionic liquid, or recycled ionic liquid
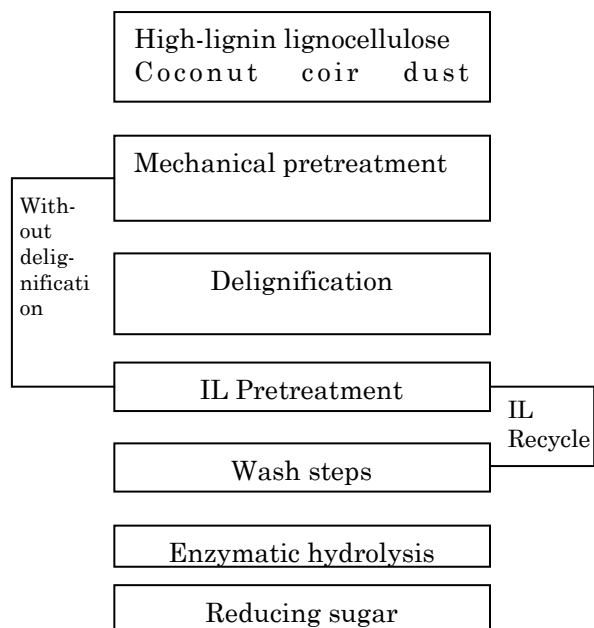

Figure 1. Diagram of the process, with deli nification (using NaOH) and non-delignification in the preparation of reducing sugar hydrolyzed from treated-coconut coir dust.

Table 1. Amount of reactants and IL product in identical molar reaction

Quantity	(tmp)	(mim)	(mmim) (dmp)
Mol	1	1	1
Molecule mass (g/mol)	140.08	82.19	222
Density (g/ml)	1.20	1.03	1.24
Mass (g)	140.08	82.19	222
Volume (ml)	118.21	80.60	178.46

[mmim][dmp] was added to 6 grams coconut coir dust, and the temperature was increased to 120 °C for 15 h. After dissolution, anti-solvent (ethanol) was mixed into the solution to recover the solids, cellulose and hemicellulose. The residue was screened with Whatman filter paper via reduced pressure and washed with hot water to remove the ionic liquid/ethanol remnants. The wastewater, which containing ionic liquid and ethanol after pretreatment, was separated their components based on differential boiling points using the simple distillation.

The efficiency of the recycled ionic liquid was calculated as the ratio the recycled ionic liquid and new ionic liquid volume. The recycled ionic liquid was reused twice for coconut coir dust pretreatment and followed by wash steps as described previously.

The combination method, NaOH+R*IL, or NaOH+IL as follows: alkaline-treated substrate was mixed with fresh- or recycled-ionic liquid for dissolution of cellulose followed by the precipitation and also wash steps, whose procedure was similar to previous method.

After pretreatment conducted, the chemical composition of coconut coir dust was analyzed by using the Chesson method [19]. This method was to carry out a comparative calculation of chemical compositions of cellulose, hemicellulose, lignin and other components of treated substrates and control (without pretreatment).

An X-ray diffraction (*Philips X'Pert X-Ray Diffractometer*) was performed to study the crystalline orientations both of treated and untreated coconut coir dust, that analysis of the XRD pattern of peaks referred to described techniques [20].

2.2. Enzymatic Hydrolysis

Enzymatic hydrolysis of pretreated- and unpretreated-substrates was conducted by four times sampling each point that procedure adapted and followed as reported previously [10-12, 21]. The substrate (1 gram, 120 mesh) was added into a suspension solution, sodium acetate buffer (pH 3, 30 ml, 0.1 M) inside an Erlenmeyer reactor.

The incubation of suspension solution took place in an oil bath at 60 °C under medium stirring for 48 h. The enzymatic reaction, which converted substrate into reducing sugar, was initiated when 0.2 ml pure cellulase from *A. niger* (Sigma-Aldrich,St. Louis, MO, USA) was added into suspension. The pH was set to 3.0 with adding sodium citrate or citric acid. Two 0.2 ml sugar liquids were sampled periodically and mixed with 1.8 ml pure water and 3.0 ml

DNS solution. The amount of sugar was measured by the DNS method. The second step was to conduct an enzymatic hydrolysis of IL-treated substrates and R*IL-treated substrates using mixture of pure cellulase+xylanase that procedure as described previously.

The proportion of the various fractions (glucose, xylose and lactose) was determined by HPLC with a Waters 1515 Isocratic HPLC pump, 2414 refractive index detector and Aminex HPX87P column (Bio-Rad, CA). Resin ionic form: hydrogen and support: sulfonated divinyl benzene-styrene copolymer 8% cross linkage. Particle size: 9 micro-meter, temperature 80 °C, mobile phase: pure water and flow rate was of 0.6 ml/min. Standard concentration (in g/l) for STD-P1, STD-P2 and STD-P3 were glucose: 5.023, 2.511, 1.256; Xylose: 5.014, 2.507, 1.253; and galactose: 5.051, 2.525, and 1.263.

3. Results and Discussions

3.1. Characterization of the ionic liquid [mmim][dmp]

The structural confirmation and chemical bonds of fresh ionic liquid [mmim][dmp] were verified by NMR using solvents, chloroform and DMSO as shown in Figure 2 and Figure 3. The ^{1}H NMR spectra exhibited average chemical shifts of d=[9.68(NCHN), 7.54 (NCHCHN), 3.75 (H_3CNCHNCH$_3$), 3.24 (P(OCH$_2$OCH$_3$)$_2$)] ppm.

FTIR diagram displayed ionic liquid structure with frequencies: 3444.00 /cm, =C−H; 1577.50 and 1385.60 /cm, C=N; 748.246 /cm, imidazole ring; 1520.60-1575.50 /cm, P=O; (1043.33-1226.50) /cm, P-OR as shown in Figure 4. Some authors have studied the chemical shifts and frequencies in that figure as previously published [14, 18].

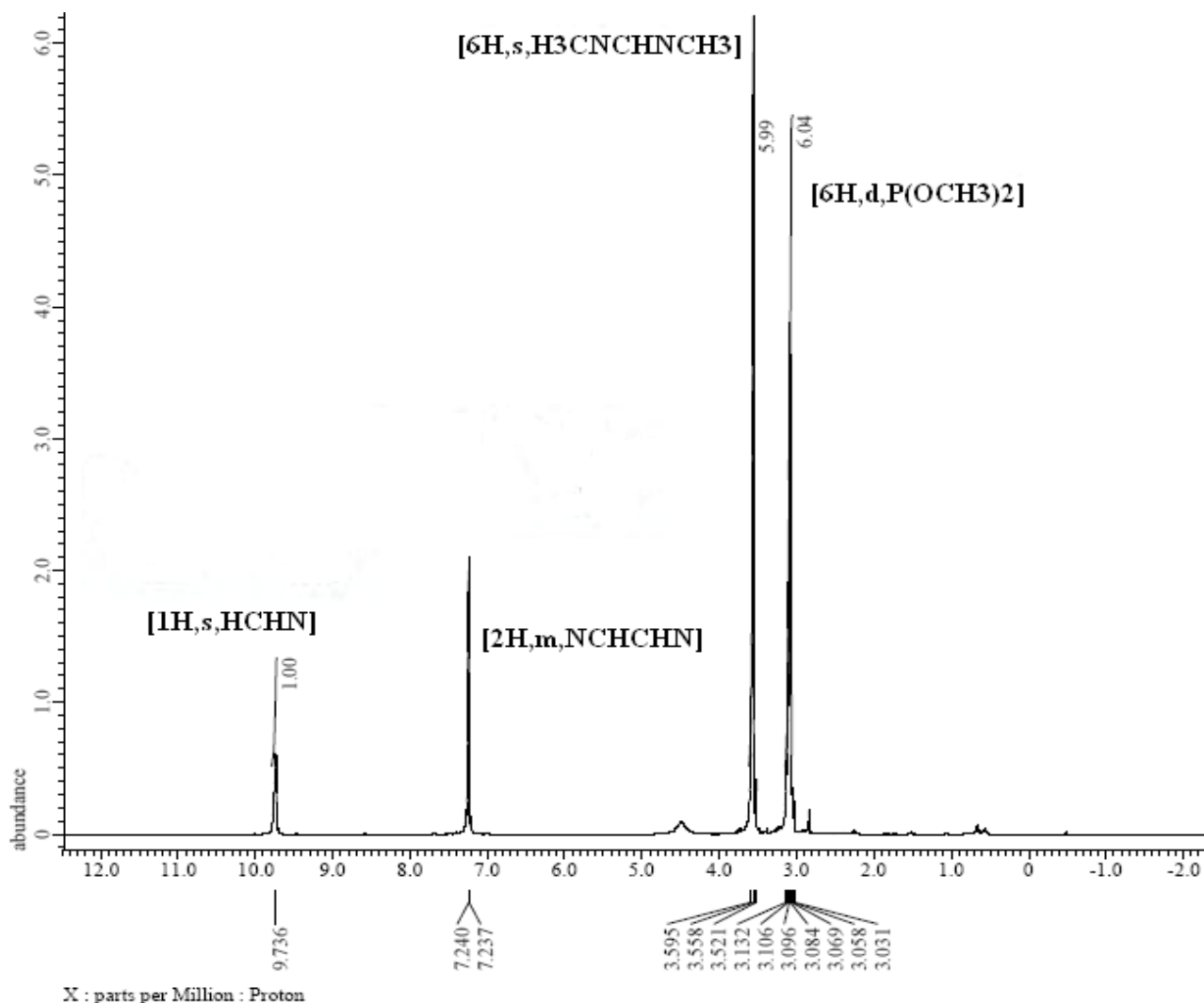The recycled ionic liquid [mmim][dmp] also was verified by NMR employing DMSO solvent

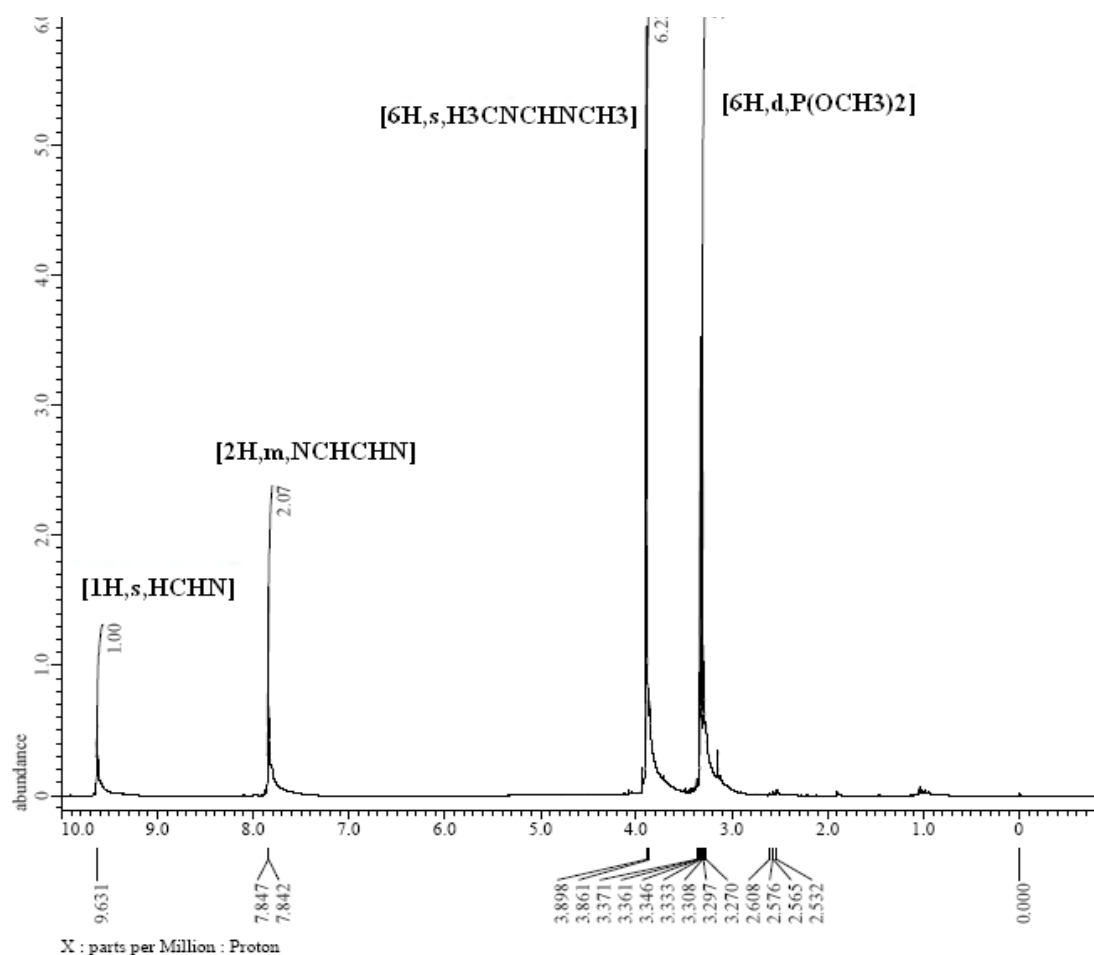**Figure 2.** NMR Spectra of ionic liquid [mmim][dmp] using chloroform-D solvent

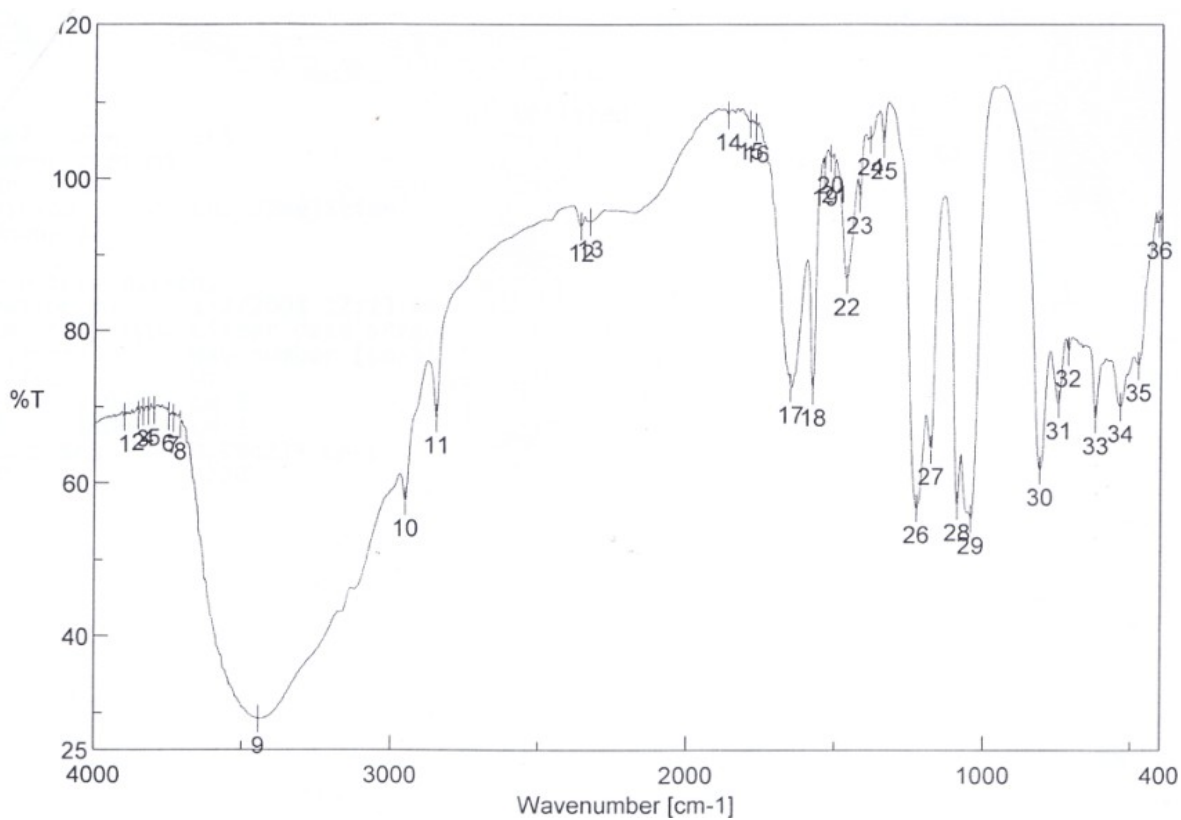**Figure 3.** NMR Spectra of ionic liquid [mmim][dmp] using DMSO-D$_6$ solvent

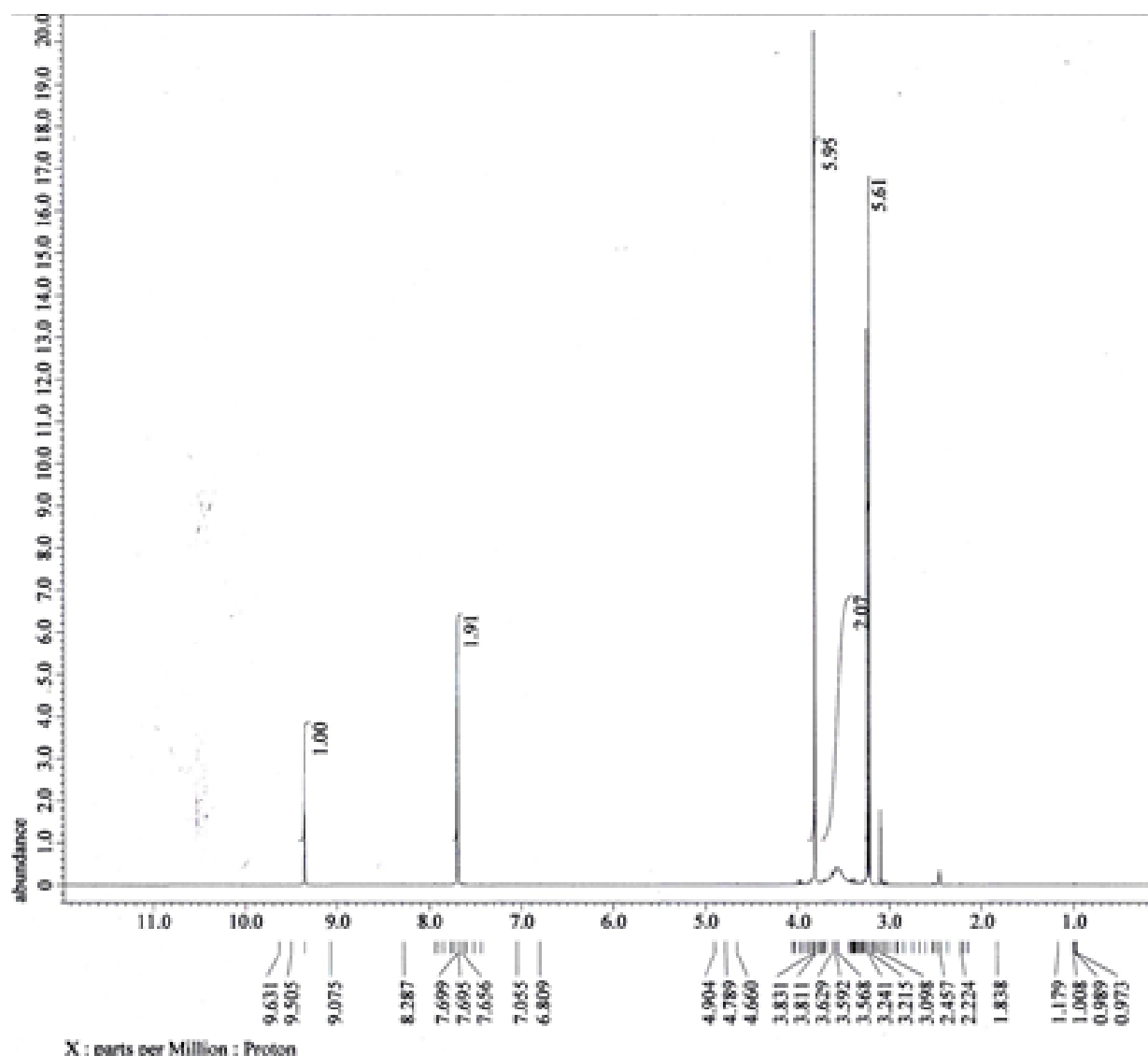**Figure 4.** FTIR spectrum of 1,3-methylmethylimidazolium dimethyl phosphate

Figure 5. NMR Spectra of recycled ionic liquid [mmim][dmp] using DMSO-D$_6$ solvent

Table 2. Chemical shift of NMR Spectra of fresh- and recycled-ionic liquids used in this work.

Substance	Group	Chemical shift (ppm)	Note
Fresh IL	(1H,s,NCHN),	9.68	Average values of chloroform and DMSO solvents
	(2H,m,NCHCHN),	7.54	
	(6H,s,H3CNCHNCH3	3.75	
	(6H,d,P(OCH2 OCH3)2)	3.24	
Recycled IL	(1H,s,NCHN),	9.35	DMSO solvent
	(2H,m,NCHCHN),	7.69	
	(6H,s,H3CNCHNCH3	3.81	
	(6H,d,P(OCH2 OCH3)2)	3.21	

as shown in Figure 5. The chemical shifts of the ^{1}H NMR spectra of recycled ionic liquid were: d=[9.35 (NCHN), 7.69 (NCHCHN), 3.81 (H$_3$CNCHNCH$_3$), 3.21 (P(OCH$_2$ OCH$_3$)$_2$)] ppm.

Table 2 shows the comparison of NMR chemical shifts of groups in fresh- and recycled-

ILs (mmim)(dmp) used in this work. These data show the spectra of chemical shifts of fresh- and recycled ionic liquids are very close each other. However, there was other broad peak appearing between peaks of [H$_3$CNCHNCH$_3$] and [P(OCH$_2$ OCH$_3$)$_2$] in recy-

Table 3. The chemical composition of coconut coir dust after various pretreatment methods

| Pretreatment	Component	Run 1 (%)	Run 2 (%)	Run 3 (%)	Run 4 (%)	Average (%)	Error (%)
Control	hemicellulose	17.87	-	17.61	17.71	17.73	0.13
	cellulose	26.68	-	26.60	26.89	26.72	0.15
	lignin	41.20	-	41.17	41.19	41.19	0.02
	others	14.24	-	14.63	14.21	14.36	0.23
NaOH	hemicellulose	19.68	20.37	20.54	20.81	20.35	0.59
	cellulose	37.68	43.44	43.72	42.39	41.81	3.44
	lignin	26.33	25.43	25.16	25.06	25.49	0.71
	others	16.35	10.77	10.59	11.74	12.36	3.32
IL	hemicellulose	25.07	-	25.29	25.35	25.24	0.15
	cellulose	21.88	-	20.35	21.80	21.34	0.86
	lignin	45.93	-	43.49	42.77	44.06	1.65
	others	7.12	-	10.87	10.08	9.36	1.98
NaOH+IL	hemicellulose	19.84	19.84	19.41	19.58	19.67	0.26
	cellulose	37.88	41.88	43.42	44.69	41.97	3.62
	lignin	28.97	28.97	25.93	26.88	27.69	1.88
	others	13.30	9.30	11.24	8.86	10.68	2.49
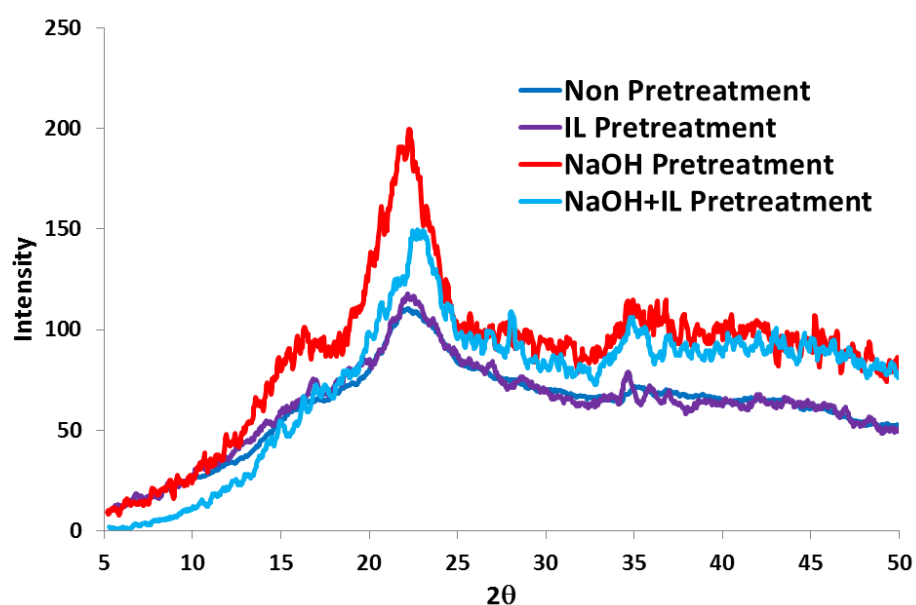
**Figure 6.** XRD pattern of substrates pretreated by NaOH, IL, NaOH+IL and without pretreatment.

cled IL, which did not exist in the fresh ionic liquid spectra. It could be lignin, which dissolved into ionic liquid. Actually, lignin has complicated NMR signals since it has many groups. However, the dominant NMR signals of lignin are assigned to protons in C-H of methoxyl groups that have chemical shift 3.70-3.81 ppm [23,24]. Even though there were impurities, recycled ionic liquid was still good enough to conduct pretreatment of biomass.

3.2. Characterization of Pretreated Coconut Coir Dust

An XRD characterization was performed to analyze the crystalline structure of substrates. In this investigation, XRD patterns were investigated at distinct three angles, $2\theta = 16.00^\circ$, 22.00°, and 35.00°, which correlated to reflector planes of (101), (002) and (040) in the cellulosic crystal as shown in Figure 6 [20-22,25-26].

After pretreatment with one percent alkaline (1% NaOH) and recovery, the diffraction pattern of coconut coir dust was typical of cellulose I, whose the properties are nearby to those of original cellulose, with a high structure and very clear peaks at the angles (16.00°, 22.00° and 35.00°) as explained above. These findings were close and comparable to previously reported studies [20, 27-29]. The improvement in the crystallinity of substrate pretreated with alkaline condition was due to the dissolution of lignin and hemicellulose.

Generally, lignin, which acts as a shield of cellulose is connected via OH groups each other. Alkaline solution can dissolve lignin so that cellulose is exposed as follows [30]:

$$\text{fiber-OH} + \text{NaOH} \rightarrow \text{fiber-O-Na}^+ + \text{H}_2\text{O} \quad (2)$$
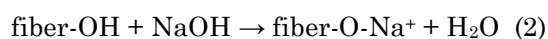
After alkaline followed by ionic liquid pretreatment (NaOH+IL), the peak (002) declined significantly, and the peak (101) almost diminished, relating to the change of the substrate from cellulose I to cellulose II, which has a low crystallinity (amorphous structure). There was also a shift of the peak, approximately 0.50° to the right, which meant that the lattice expanded after pretreatment.

The curve of IL-treated substrate overlapped with untreated substrate graph. It was indicative that ionic liquid did not work maximum to dissolve cellulose and hemicellulose because of high-lignin substrate.

The ionic liquid, high polarity, which consists of cations and anions, interacts electrically with cellulose. In this process, NaOH-treated cellulose was dissolved in ionic liquid [mmim][dmp] and its crystalline structure was modified to amorphous structure, cellulose II. The cellulose II, which was modified to amorphous structure was easy attacked by enzyme in hydrolysis process producing fermentable sugars, so the yield of sugar improved significantly as previously published authors [7, 14, 27, 31].

Chesson method was performed to analyze the compositions of cellulose, hemicellulose, and lignin in coconut coir dust [19]. Table 3 shows the chemical compositions of substrates after pretreatment and without pretreatment, as control.

The chemical contents of untreated coconut coir dust were of 26.72 percent cellulose, 17.73 percent hemicellulose and 41.19 percent lignin. After pretreatment, some lignin was removed significantly. The highest lignin removal was found that after coconut coir dust pretreated by NaOH and NaOH+IL. In contrast, pretreatment of IL showed the lowest lignin removal, which was relatively similar to control. The highest content of cellulose was obtained from NaOH pretreatment followed by ionic liquid at of 41.97 percent and the lowest cellulose obtained from the ionic liquid pretreatment was of 21.34 percent. The content of hemicellulose obtained from the ionic liquid pretreatment was of 25.24 percent.

Results indicated that ionic liquid could not dissolve maximum nor could transform the crystalline structure of substrate, which has high lignin, to amorphous for lignin impeded the electrostatics interaction between ionic liquid and cellulose to form uniform solution. Based on this finding, it was necessary the high-lignin lignocellulose was treated firstly by alkaline to dissolve lignin and then was proceeded ionic liquid pretreatment.

Other report showed the chemical composition of coconut coir without pretreatment is different slightly compared to that present work. The origin and maturity level influenced to the chemical composition of coconut coir lignocelluloses [32].

The present work that the chemical compositions of recycled ionic liquid-pretreated substrates were not conducted. The current report, however, showed the chemical compositions of substrates pretreated by recycled ionic liquid and fresh ionic liquid were not different significantly as previously published study [15].

3.3. Enzymatic hydrolysis of coconut coir dust pretreated by recycled ionic liquid [mmim][dmp] using a single cellulase

This section is to report the effect of recycling of IL [mmim][dmp] on pretreatment of coconut coir dust and compared to that of fresh IL, alkaline and their combination. The recycling of ionic liquid is necessary that aims to reduce the cost of sugar manufacturing as the price of the ionic liquid is still expensive.

The technique of ionic liquid recycling was simple by using the simple distillation apparatus and operating at temperature of 120 °C. Table 3 shows the efficiency of recycled ionic liquid after being used on the pretreatment of coconut coir dust for ionic liquid and NaOH+IL methods. It was found that the efficiency of recycled ionic liquid (R*IL) after being separated from waste of IL and NaOH+IL pretreatments of coconut coir dust, was still high, 78.0-98.5 percent. While, the other report showed that the efficiency of recycled IL was of 85-90 % [9, 15].

The recycled ionic liquid was reused for pretreatment and was continued with enzymatic hydrolysis. The recycled ionic liquid was reused twice for pretreatment coconut coir dust and that result was compared to fresh ionic liquid pretreatment.

The enzyme preparation and calculation of standard curves for glucose, xylose and total reducing sugar followed and adapted from the standard method [33]. The first investigation was the treated substrates and untreated substrate hydrolyzed to sugar using single cellulase for 48 h. The TRS (total reducing sugar) concentration obtained from coconut coir dust pretreated with fresh IL was 2.74 g/l, higher than that of the non-pretreated substrate at 2.10 g/l as shown in Table 5 and Figure 7.

When recycled ionic liquid employed in pretreatment, there was no significant difference between the TRS converted from substrates pretreated with the first recycled IL (1R* IL) or the second recycled IL (2R*IL) and fresh IL. The amount of sugar released from 1R*IL-, 2R*IL-treated substrate was 2.15 g/l decrease

slightly 7.41 percent from fresh IL-treated substrate recorded at 2.74 g/l.

The results showed that fresh, or recycled ionic liquid pretreatment could not increase significantly the digestibility of enzymatic hydrolysis since the lignin was still connected the cellulose and hemicellulose as source of sugars, which related with XRD analysis and chemical composition of substrates.

The TRS concentration of regenerated coconut coir dust increased significantly after pretreatment combination, NaOH+IL and NaOH+R*IL as shown in Figure 8. The amount of TRS liberated from NaOH+IL- and NaOH+1R* IL-pretreated substrates were 4.00 g/l and 3.86 g/l, respectively, and the TRS released from NaOH+2R*IL-pretreated substrate was 3.77 g/l.

The amount of sugar released enzymatically, increased after substrates were treated by NaOH+IL-, or NaOH+R*IL, whose crystalline structure was amorphous, cellulose II which was easy attached by enzymes. Alkaline pretreatment caused dissolution of lignin so cellulose exposed, therefore ionic liquid dissolved cellulose easily to modify the substrate.

The first yield of enzymatic hydrolysis is defined as mass of total reducing sugar obtained, TRS (in gram) per mass of dried coconut coir dust, CCD (in gram). Mass of TRS was calculated by using formula TRS concentration multiplied by suspension volume. The second yield is ratio between mass of TRS and mass of T TRS (theoretical total reducing sugars) obtained from the chemical composition (cellulose+hemicellulose) of native coconut coir dust. For original coconut coir dust, T TRS was

Table 4. Efficiency of recycled ionic liquid, the first recycled ionic liquid (1R*IL) and the second recycled ionic liquid (2R*IL)

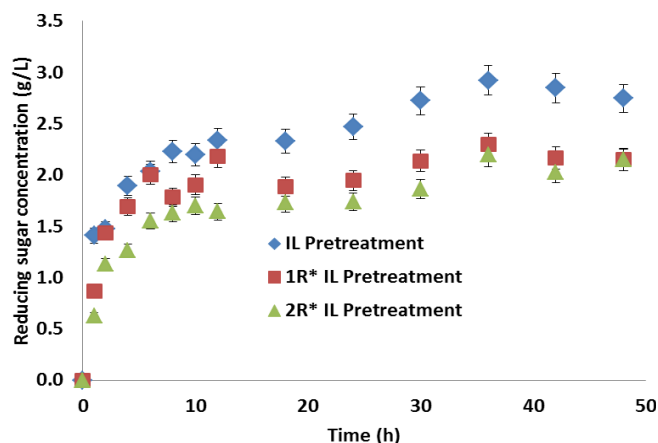| Pretreatment	Efficiency		
	Initial	1R*	2R*
IL	100	98	78
NaOH+IL	100	98.5	91.5

Figure 7. Time course of the effect of pretreatment methods on enzymatic hydrolysis (60 °C for 48 h) using single cellulase of coconut coir dust pretreated by fresh IL, [mmim][dmp], the first recycled, 1R* IL and the second recycled, or 2R* IL at 120 °C for 15 h.

0.26 multiplied by the weight of the dried CCD plus 0.17 multiplied by the weight of dried CCD.

HPLC measurement was performed to verify the result from DNS measurement and to analyze the type of reducing sugar which converted from substrates. The sugars obtained were dominated by glucose, xylose and galactose.

Table 5 describes the yields of sugars measured by HPLC and DNS resulted from enzymatic hydrolysis of recycled IL- fresh IL-, NaOH-pretreated substrates and their combination with alkaline, NaOH+IL, NaOH+R*IL, using single cellulase and mixture of cellulase+xylanase for 48 h.

Generally, the yield or amount of sugar measured by DNS is higher than that of HPLC method. DNS method measures the total content of sugars in liquid without informing types of sugar. While, HPLC measurement depends on its sensor sensitivity. In this study, HPLC equipment used was only capable detecting three sugars-glucose, galactose and xylose so it resulted the lower sugar concentration compared to that performed with DNS. As an example, the amount of sugar derived from untreated substrate (control) using HPLC was 0.88 g/l lower than that performing DNS method recorded at 2.10 g/l.

The yield of sugar released from IL-treated substrate was 0.11 g sugar / g cellulose+hemiselulosa, or 0.05 g sugar / g dried substrate measured HPLC. When DNS method applied, the yield of sugar was 0.19 g sugar / g cellulose+hemicellulose for the similar substrate.

Enzymatic hydrolysis of 1R* IL- and 2R* IL-pretreated substrates resulted similar yield at 0.06 g sugar / g dried lignocellulose or 0.15 g sugar / cellulose + hemicellulose using DNS, while HPLC method gave yields 0.13 and 0.12 g sugar / cellulose + hemicellulose for the same substrates.
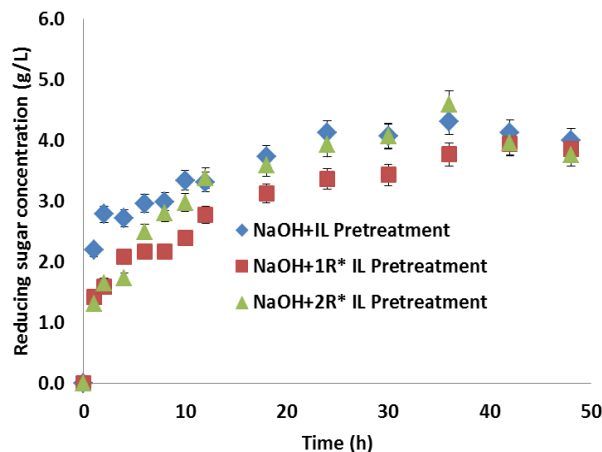

Figure 8. Time course of the effect of pretreatment methods on enzymatic hydrolysis (60 °C for 48 h) introducing single cellulase of coconut coir dust pretreated by alkaline followed by fresh IL (NaOH+IL), alkaline followed by the first recycled IL (NaOH+1R* IL) or alkaline followed by the second recycled IL (NaOH+2R* IL) at 120°C for 15 h.

Table 5. The yield and HPLC measurement of enzymatic hydrolysis using a single cellulase and mixture of cellulase+xylanase of recycled IL-pretreated substrate, compared with those of IL-pretreated, NaOH-pretreated substrates and their combination for 48 h

	cellulase										Cellulase+xylanase		
	Total reducing sugar concentration (g/l) HPLC				HPLC Yield		Total DNS (g/l)	DNS Yield		DNS (g/l)	DNS Yield		
					(1)	(2)		(1)	(2)		(1)	(2)	
Pretreatment					g.TRS	g.TRS		g.TRS	g.TRS		g.TRS	g.TRS	
	Glu-cose	Xy-lose	Galac-tose	To-tal	g.CCD	g.T.TRS		g.CCD	g.T.TRS		g.CCD	g.T.TRS	
Control	0.83	0.02	0.04	0.88	0.03	0.06	2.10	0.06	0.15	2.72	0.08	0.19	
IL	1.26	0	0.38	1.64	0.05	0.11	2.74	0.08	0.19	3.17	0.10	0.22	
1R* IL	1.16	0.20	0.54	1.89	0.06	0.13	2.15	0.06	0.15	3.34	0.10	0.23	
NaOH	1.56	0.11	0.64	2.31	0.07	0.16	3.63	0.11	0.25	3.83	0.11	0.27	
2R* IL	1.27	0	0.52	1.78	0.05	0.12	2.15	0.06	0.15	2.54	0.08	0.18	
NaOH+IL	1.32	0.13	0.62	2.07	0.06	0.14	4.00	0.12	0.28	4.64	0.14	0.32	
NaOH+1R*IL	1.72	0.16	0.68	2.56	0.08	0.18	3.86	0.12	0.27	3.87	0.12	0.27	
NaOH+2R*IL	1.59	0.17	0.63	2.38	0.07	0.17	3.77	0.11	0.26	3.55	0.11	0.25	

The sugars liberated from NaOH+IL-, NaOH+1R*IL- and NaOH+2R*IL-pretreated substrates generated yield 0.28, 0.27, and 0.26 g sugar/g cellulose+hemicellulose, respectively. HPLC measurement resulted 0.14, 0.18 and 0.17 g sugar / g cellulose+hemicellulose.

The data show that the yields of sugar released from substrate treated by recycle ionic liquid, or combination of alkaline and recycled ionic liquid were relatively still as high as compared to that treated by fresh IL and NaOH followed by new IL.

Other research demonstrated that the yields of enzymatic hydrolysis of IL-pretreated substrates and recycled-treated substrates differed slightly 2-10 percent [15]. When recycled IL treated on wheat straw the yield of sugar changed very small at 0.1 percent from that fresh ionic liquid pretreatment observed [34].

This study also showed that the optimum method for high-lignin substrate was alkaline followed by IL (NaOH+IL) and alkaline followed by recycled IL (NaOH+R*IL), which gave the highest yield of sugar. This results correlated with structure of NaOH+IL-treated substrate, cellulose II (amorphous) which was very easy digested by enzymes converted to sugar.

3.4 Effect of recycled IL on enzymatic hydrolysis of coconut coir dust using the mixture of cellulase and xylanase

This study also conducted an analysis the effect of recycled IL [mmim][dmp] on enzymatic

hydrolysis of substrate using mixture of cellulase and xylanase.

Figure 9 shows the effect of pretreatment methods on enzymatic hydrolysis of coconut coir dust pretreated by fresh ionic liquid, first recycled and the second recycled ionic liquid employing the mixture of cellulase and xylanase for 48 h, which were also shown in Table 5.

The total reducing sugar concentration obtained from enzymatic hydrolysis of substrate pretreated by new ionic liquid was 3.17 g/l as the yield was 0.22 g sugar / g cellulose+hemicellulose. If substrates pretreated by 1R* IL and 2R* IL, the total reducing sugar liberated from substrates were of 3.34 g/l and 2.54 g/l, whose yields were 0.23 and 0.18 g sugar / g cellulose+hemicellulose, respectively.

The effect of pretreatment methods on enzymatic hydrolysis using mixture of cellulose+xylanase of coconut coir dust pretreated by NaOH+IL, NaOH+1R*IL or NaOH+2R* IL at 120 °C is shown in Figure 10. The sugar released enzymatically from substrate pretreated by alkaline followed by new ionic liquid, NaOH+IL was of 4.64 g/l that was equal to yield 0.32 g sugar / g cellulose+hemicellulose.

The substrates treated by NaOH+1R*IL and NaOH+2R*IL produced sugar 3.87 g/l, 3.55 g/l, respectively, which declined slightly from previous method using alkaline and fresh ionic liquid. The yields of digestibility of these recycled IL-treated substrates were 0.27 and 0.25 g
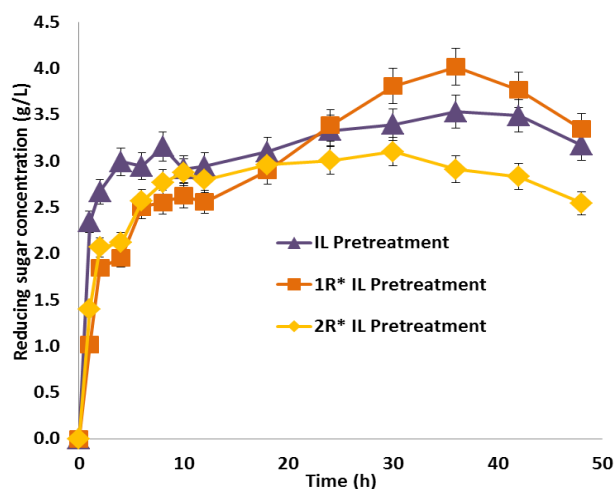

Figure 9. The effect of pretreatment methods on enzymatic hydrolysis (60 °C for 48 h) employing mixture of cellulase+xylanase of coconut coir dust pretreated by fresh IL [mmim][dmp], the first recycled IL, 1R* IL and the second recycled IL, or 2R*IL at 120°C for 15 h.
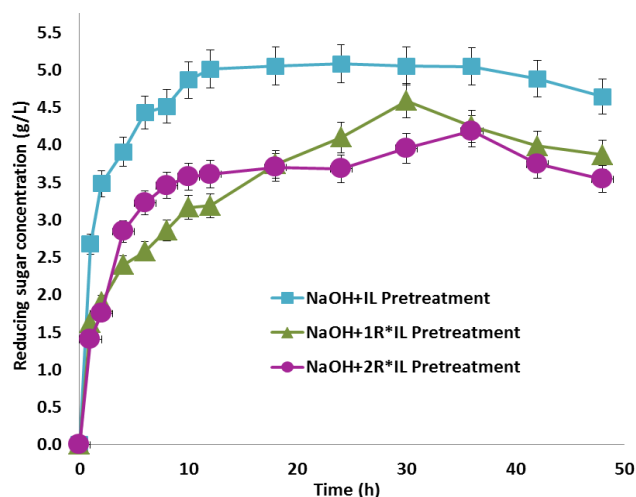

Figure 10. The effect of pretreatment methods on enzymatic hydrolysis (60 °C for 48 h) using mixture of cellulase+xylanase of coconut coir dust pretreated by alkaline followed by fresh IL (NaOH+IL), alkaline followed by the first recycled IL (NaOH+1R* IL) or alkaline followed by the second recycled IL (NaOH+2R* IL) at 120°C for 15 h.

sugar/g cellulose+hemicellulose, which decreased small as the numbers of recycling increases which was comparable to reported authors [15].

Generally, TRS hydrolyzed from treated substrates using mixture of enzyme is slightly higher than that using single enzyme. The single cellulase only attacks cellulose chain to liberate glucose, galactose sugars, while the mixture of enzymes (cellulase+xylanase) work together; cellulase attacks cellulose to release glucose, galactose and xylanase trims hemicellulose to produce xylose sugars [35].

This study emphasized on the effect of recycled ionic liquid pretreatment on coconut coir dust. The results obtained that there were no significant differences of amount of sugar liberated from coconut coir dust pretreated by recycled ionic liquid and new ionic liquid. The same trend was shown by NaOH+IL-substrate and NaOH+R*IL-substrates, whose they liberated amount of sugars with small differences as previously described.

For recycled ionic liquid pretreatment, these results are similar to previous work as described reports [15, 34, 36]. The high-lignin lignocellulose, such as coconut coir dust, produced less sugar than other lignocellulosic materials, such as sugarcane bagasse or poplar wood, that has lower lignin contents [34-39].

This invention shows that the recycled ionic liquid, which used for biomass pretreatment, is critical option to reduce the production cost of reducing sugar and bioethanol for industrial scale, which will be elaborated in sub section 3.5.

3.5. Economic analysis of consumables cost drivers

The pretreatment of biomass is the most important step of overall stages in sugar production. Pretreatments using ionic liquid has attracted recently since their advantages to modify the crystalline structure and to dissolve lignin enhancing enzymatic hydrolysis [27-29]. However, IL based pretreatment is necessary to improve and then to know some cost drivers in order to reduce the production cost and to realize the industry scale.

In this section was only to analyze of cost driver variables, consumable expenditure including electricity, contributing to the overall cost of sugar preparation converted from coconut coir dust pretreated by recycled IL, fresh IL, alkaline and their combination. It would understand about percentage distribution of cost driver variables in the lab scale and would be base line for the next industry scale. This study assumed that all consumables used were industry grade produced by China companies, which were available via web www.alibaba.com/trade.

The present study, IL-based method was employed by using "water-wash" (WW) path, whose enzymatic hydrolysis was free-ionic liquid. Prior to enzymatic hydrolysis, the IL-treated substrate should be washed using water until it was neutralized [40, 41]. This process, modified WW path, was used two processes, delignification (NaOH) and without alkaline pretreatments of biomass as shown in Fig. 1.

To reduce the production cost of reducing sugar, IL was synthesized ourselves reacting [mim] and [tmp] prior to conducted pretreatment and enzymatic hydrolysis as described previously. The price of industrial grade consumables, electricity, cost per kg biomass and percentage of cost drivers were shown in Table 6. In the present work, lab scale, 160 grams of water were used to wash 1 gram of treated biomass. The ionic liquid employed on substrate pretreatment was 21 ml per 1 gram coconut coir dust. One gram enzyme was introduced to prepare 100 ml buffer, or 1/20 gram enzyme per one gram treated biomass. The mass of alkaline used in experiment was 0.2 gram per 1 gram untreated biomass.

The price of reactants to synthesize ionic liquid product is still expensive. In this analysis found that the recycle of ionic liquid is the most important of preparation steps of fermentable sugars, which contributed on the highest reduction of the cost.

The cost of (mim) reactant was US$ 7.77 per kg biomass, which the highest percentage of cost driver of 88.58 percent. On the WW path, usage of water was much and then contributed on production cost at US$ 0.032 per kg biomass, or 0.36 percent of cost driver. Electricity generated the cost driver was at 6.02 percent, which was the second place of cost drivers, or it was US$ 0.53 per kg substrate. The total cost of consumables expenditures, including electricity found was US$ 8.77 per kg for NaOH+IL-treated lignocellulose.

Table 7 shows the total cost per kg biomass and the total cost per kg sugar hydrolyzed from coconut coir dust pretreated by new IL, recycled-IL, alkaline and their combination based on yield (2) using mixture of enzymes. The total cost per kg biomass was calculated from summing the cost of consumables per kg biomass contributing the overall cost. For example, total cost per kg biomass of control, or without pretreatment, was only US$ 0.79, which the result

of expenditures summing of citrate acid, sodium citrate, enzymes and electricity. While the total cost per kg sugar was obtained by dividing the total cost per kg biomass with yield (2) of sugar multiplied by mass of cellulose+hemicellulose,

The highest cost discovered was US$ 91.79 per kg sugar (or US$ 8.68 per kg biomass) for fresh IL pretreatment and the second place was US$ 63.74 per kg sugar of NaOH+IL-treated substrate. When recycled IL employed, the cost declined extremely, which was similar to other authors report [36]. The 1R* IL- and 2R* IL-treated substrates generated the production cost US$ 8.27, US$ 10.57 per kg sugar, respectively. The production costs of sugar hydrolyzed from NaOH+1R*IL- and NaOH+2R*IL-treated substrates were US$ 7.80, US$ 8.43 per kg sugar, respectively, assuming ethanol recovery 100%.

The extreme decrease of cost production was caused by no expenditure of reactants, (mim) and (tmp), which mostly dominated of the cost.

While substrate treated by alkaline, as one of conventional methods, the cost was US$7.80 per kg sugar, or US$ 0.91 per kg biomass. Although alkaline pretreatment gave low production cost of fermentable sugar, it could not be recycled.

These findings revealed that the reduction cost of sugar preparation hydrolyzed from high-lignin substrate pretreated by IL depended on how many conducting the recycling of ionic liquid and how reducing of reactant (mim) price

that mostly dominating the all of cost drivers. In laboratorium scale, the preparation of reducing sugar hydrolyzed from coconut coir dust treated by fresh ionic liquid or combination with alkaline, was not feasible.

However, by recycling the ionic liquid, and increasing the capacity, the cost of sugar production would decrease significantly and that could be considered for the next scale up as previously reported [40, 41]. It was identified that the cost drivers contributed on sugar production converted from coconut coir dust mostly were (mim), electricity, citrate acid and (tmp) reactant. In the next work, the critical option to

Table 7. Total cost/kg biomass and cost per kg sugar hydrolyzed from coconut coir dust of various pretreatments in laboratorium scale with yield (2) basis using mixture enzymes

No	Pretreatments	Cost /kg biomass (US$/kg)	Cost/kg sugar (US$/kg)
1	Control	0.79	9.62
2	IL	8.68	91.79
3	NaOH	0.91	7.80
4	1R*IL	0.82	8.27
5	2R* IL	0.82	10.57
6	NaOH+IL	8.77	63.74
7	NaOH+1R* IL	0.91	7.80
8	NaOH+2R* IL	0.91	8.43

Table 6. The cost per kg biomass and percentage of cost driver variables in the present work using alkaline followed fresh ionic liquid pretreatments (NaOH+IL) in Lab scale.

No	Expenditure	Price (US$/kg)*	Ratio consumables / biomass (g/g)	Cost/kg biomass (US$/kg)	Percentage of cost drivers (%)
1	MIM (Methylimidazole)	1.00	7.77	7.77	88.58
2	TMP (Trimethyl Phosphate)	0.0072	13.24	0.095	1.09
3	Ionic liquid	0.00	21	0	0
4	Sodium Hydroxide	0.44	0.2	0.088	1.00
5	Citrate acid	1.20	0.17	0.20	2.33
6	Sodium citrate	0.85	0.062	0.053	0.60
7	Enzymes (cellulase, xylanase)	0.003	0.05	0.00015	0.0017
8	Water	0.0002**	160	0.032	0.36
9	Raw material	0.00	0.00	0.00	0.00
10	Electricity **	0.00	0.00	0.53	6.02
	Total			8.77	100

Note: *FOB; ** East Java price

reduce the production cost is to recover the valuable lignin from wastewater. To minimize the electrical cost, the reactors of hydrolysis, pretreatment and recycling should be integrated and established the continuous system in order to increase the efficiency.

4. Conclusions

Reducing sugars have been successfully hydrolyzed from high-lignin lignocellulose from coconut coir dust pretreated by recycled ionic liquid, alkaline solution and their combination. The findings found that the yield, or concentration of sugar hydrolyzed from high lignin substrate pretreated by recycled ionic liquid was relatively similar to those of substrates pretreated by fresh ionic liquid. Analysis of cost driver variables found that recycling of ionic liquid was most important point to reduce the production cost of sugar from high-lignin lignocellulose, coconut coir dust.

Acknowledgments

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