Comparative Study of the Preparation of Reducing Sugars Hydrolyzed from High-Lignin Lignocellulose Pretreated with Ionic Liquid, Alkaline Solution and Their Combination

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Abstract. The ionic liquid [MMIM][DMP] was synthesized from the reactants methyl imidazole [MIM] and trimethylphosphate [TMP] and verified using ^{1}HNMR and FTIR. Coconut coir dust was pretreated with a 1% alkaline solution. Its crystalline structure increased significantly due to the dissolution of lignin and hemicelluloses under alkaline conditions, exposing the cellulose. After NaOH and IL were employed, the XRD showed that peak (002) decreased significantly and peak (101) almost vanished. This significant decrease in crystallinity was related to the alteration of the substrate from the cellulose I structure to the cellulose II structure. The pretreated substrates were hydrolyzed to convert them to reducing sugars by pure cellulase and xylanase, and the reaction was conducted at 60°C, pH 3, for 12 or 48 hours. The yields of sugar hydrolyzed from untreated and NaOH-pretreated substrates were 0.07 and 0.12 g sugar/g lignocellulose, respectively. Pretreatment with IL or the combination of NaOH+IL resulted in yields of reducing sugars of 0.11 and 0.13 g/g, respectively. These findings showed that IL pretreatment of the high-lignin lignocellulose is a new prospect for the economical manufacture of reducing sugars and bioethanol in the coming years.

1 Introduction

The cellulosic arrangement has a robust link called a β (1→4) glycosidic bond, and an H bond at the hydroxyl OH acts as a shield for glucose monomers [1]. Lignocellulosic pretreatments, including lignin degradation with chemical and physical techniques, have been carried out to transform the cellulosic structure, thus increasing the yield of sugar in enzymatic hydrolysis [2-4]. Acid techniques have been used to pretreat grape stalks in order to recover both cellulose and hemicellulose; however, the acid solution cannot be recycled [5].

The sub- and supercritical techniques are very expensive and difficult to scale up on an industrial stage because of safety and cost considerations [6].

Ionic liquid as a solvent for lignocellulosic pretreatment has been thoroughly studied due to its advantages compared to traditional solvents [7,8]. Sugarcane bagasse has been successfully hydrolyzed into reducing sugars and ethanol by applying IL 1-allyl-3-methylimidazolium chloride [[amim] Cl] pretreatment. This ionic liquid successfully liquefied sugarcane bagasse (SCB), and its crystallinity index declined by 42%, so the hydrolysis performance was enhanced [9]. Dissolving the lignocellulose into ionic liquid causes the transformation of the crystal orientation from high- to low-crystalline structures that are very easily converted into reducing sugars and then fermented into bioethanol [10-13].

This report consists of three stages as follows: synthesis of ionic liquid, pretreatment of coconut coir dust by ionic liquid, NaOH and NaOH+ionic liquid and hydrolysis of coconut coir dust into fermentable sugars. To verify the structure, NMR (*nuclear magnetic resonance*) and FTIR (*Fourier transform infra-red*) analysis was performed, followed by the pretreatment of coconut coir dust using the ionic liquid. The crystalline structures of all substrates, non-pretreated and pretreated lignocelluloses, were analyzed by XRD. Finally, the pretreated and non-pretreated coconut dust lignocellulose was hydrolyzed into reducing sugars by cellulase and xylanase. 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).

2   Materials and Methods

2.1   Material Preparation

The synthesis of the ionic liquid, *1,3-methylmethylimidazolium dimethyl phosphate*, was based on previously described methods [14-16]. The IL structure characterization was verified by ^{1}H NMR (JNE-ECS-400MHz, JEOL, Japan). The method proposed by Datta and Rathin was used to analyze the chemical composition of coconut coir dust [17]. The next stage was pretreatment of the lignocellulosic materials of coconut coir dust, using the ionic liquid generated as described in a previous study [18]. The other method was NaOH pretreatment followed by ionic liquid pretreatment of coconut coir dust followed by the precipitation and wash steps. XRD (*Philips X'Pert X-Ray Diffractometer*) was employed to analyze the

crystal structure of pretreated coconut coir dust. Analysis of the XRD pattern of peaks followed previously described methods [19].

2.2   Enzymatic Hydrolysis

Enzymatic hydrolysis was carried out as described previously [10,11,12,20]. A suspension solution was prepared by mixing sodium acetate buffer (pH 3, 30 mL, 0.1 M) and coconut coir dust (1 gram, 120 mesh) inside an Erlenmeyer reactor. The suspension solution was incubated in an oil bath at 60°C under moderate stirring for 12-48 hours. The enzymatic reaction was started by adding 0.2 ml pure cellulase from *A. niger* (Sigma-Aldrich,St. Louis, MO, USA). The pH was adjusted to 3 with sodium citrate or citric acid. Two 0.2-mL samples were removed periodically and diluted with 1.8 mL distilled water and 3 mL DNS solution. The sugar concentration was determined by the DNS technique [11-12]. 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).

3   Results and Discussion

3.1   Characterization of the Ionic Liquid [MIM][DMP]

The ionic liquid synthesis was a highly exothermic reaction characterized by an extreme increase in temperature from 115°C to 200°C in only 5 minutes. In an exothermic reaction, the formation heat of the reactants is greater than the formation heat of the products, so heat is released. These results confirm the results of previous research on the synthesis of the ionic liquids [16].

Figure 1 shows the confirmation of the structure and bondage in the ionic liquid measured by NMR with two different solvents, chloroform and DMSO. The 1H NMR characterization showed average chemical shifts of $\delta \times 10^{-6}$ [9.6835 (1H,s,NCHN), 7.5415 (2H,m,NCHCHN), 3.7465 (6H,s,H_3CNCHNCH$_3$), 3.2425 (6H,d,P(OCH$_2$ OCH$_3$)$_2$)]. FTIR verified the structure of the ionic liquid with wave constants of 3444/cm, =C-H; 1576.52 and 1385.6/cm, C=N; 748.245/cm, imidazole ring; 1520.6-1576.52/cm, P=O; (1042.34-1226.5)/cm, P-OR, as shown in Figure 2(a). Other studies have described the chemical shifts and wave constants in that figure [16,18].
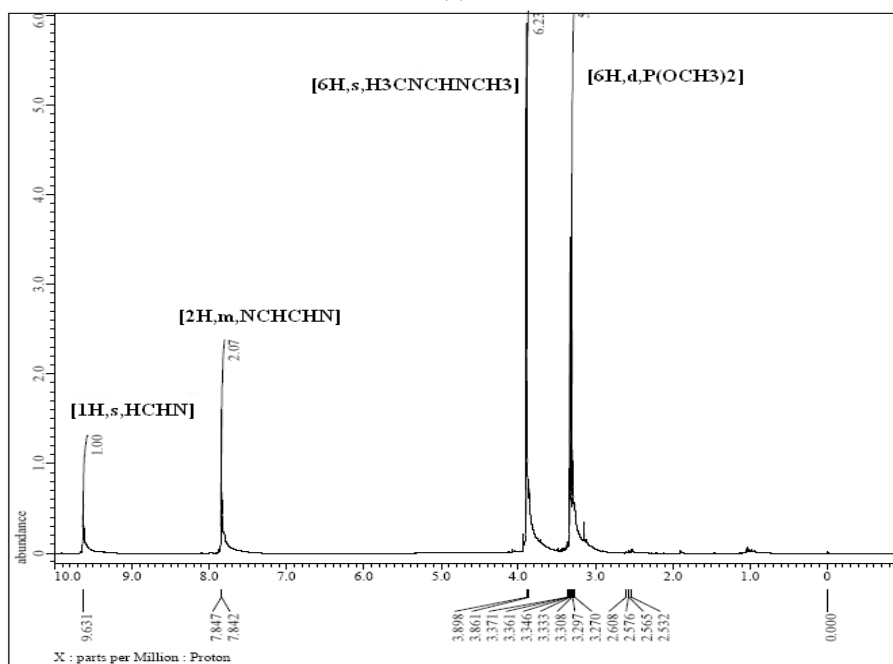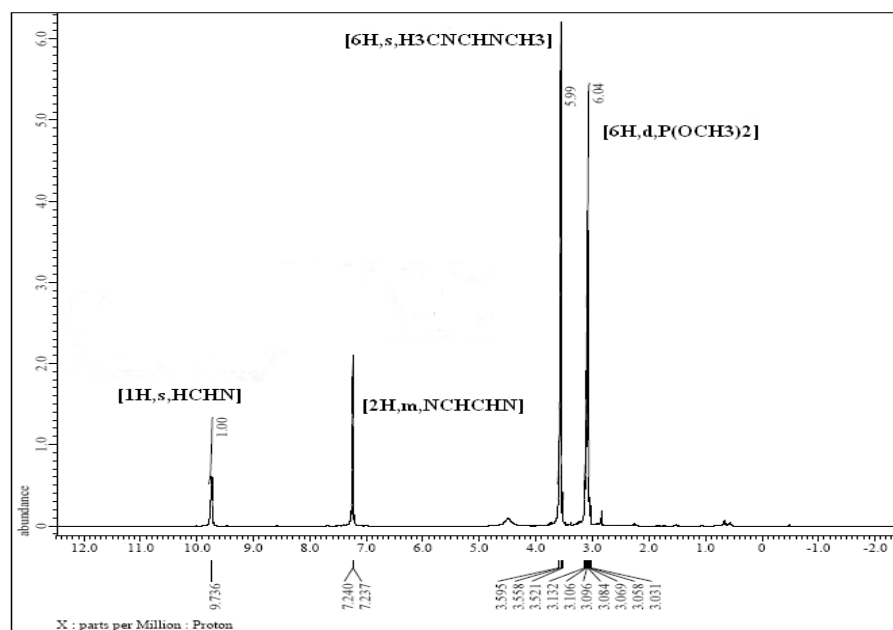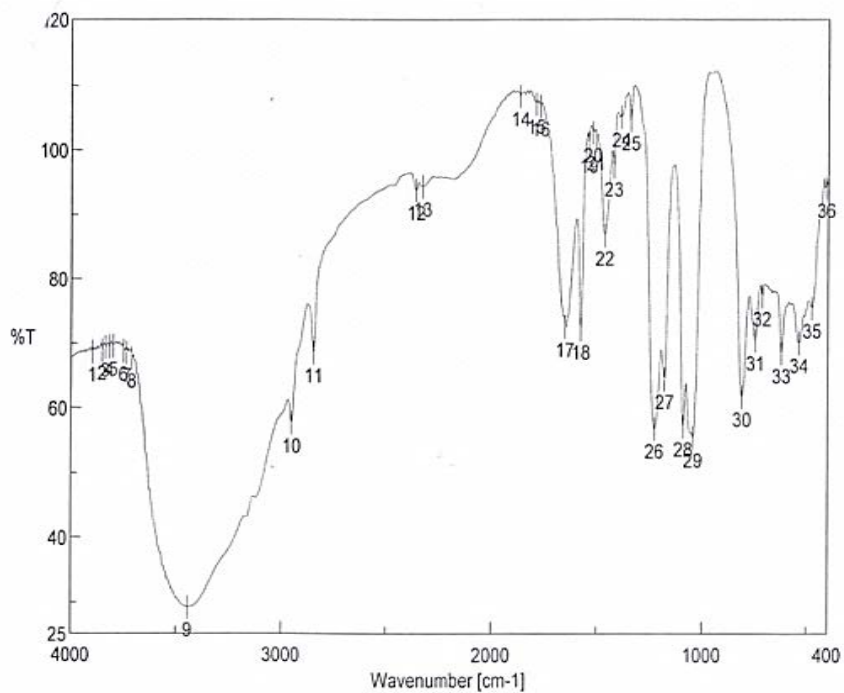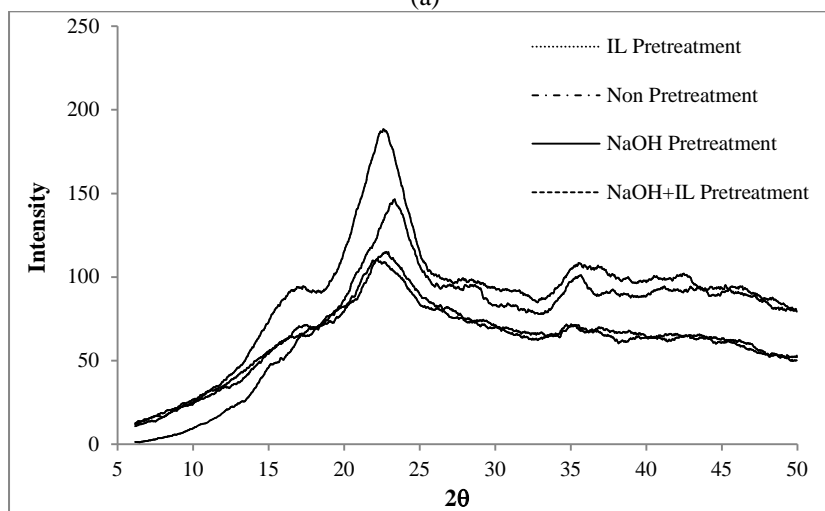
Figure 1 NMR Spectra of *1,3-methylmethylimidazolium dimethyl phosphate* with chloroform-D solvent (a) and DMSO-D$_6$ solvent (b).

(a)

(b)

Figure 2 (a) FTIR spectrum of *1,3-methylmethylimidazolium dimethyl phosphate* and (b) XRD pattern of coconut coir dust for three pretreatments and without pretreatment.

3.2   Characterization of Pretreated Coconut Coir Dust

In this study, XRD diffraction peaks were observed at three angles, $2\theta = 16^o$, 22°, and 35°, which related to planes of (101), (002) and (040) in the lattice, as shown in Figure 2(b). These are the typical patterns for crystalline cellulose I, which have been studied by other authors [19,21-23]. After pretreatment with 1% NaOH and regeneration, the diffraction intensity of substrates was typical of cellulose I. Cellulose I properties are close to those of cellulose, with a high crystallinity and distinct peaks at the angles (16°, 22° and 35°) described above. These results were similar and comparable to previously published reports [19-23]. The increase in the crystalline index of substrate pretreated with NaOH was mainly caused by the liquefaction of the amorphous parts (lignin and hemicellulose). After pretreatment with NaOH+IL, the peak (002) decreased significantly, and the (101) plane almost disappeared, corresponding to the transformation of the material from cellulose I to cellulose II, which has an amorphous structure [18,24,25].

Table 1   The chemical composition of coconut coir dust after various pretreatment methods.

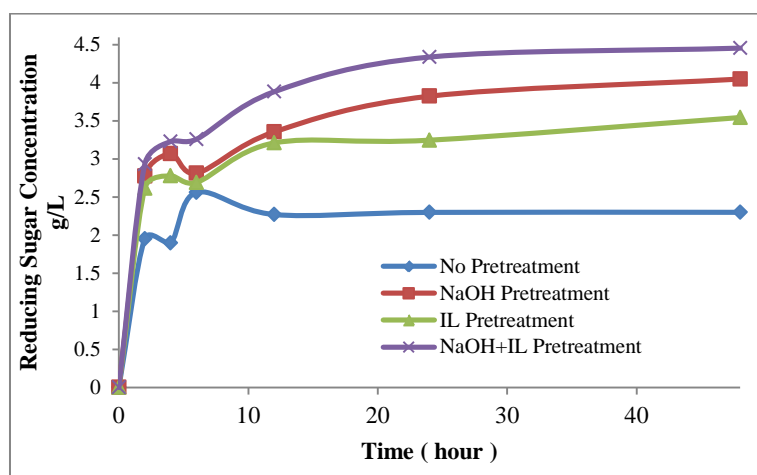| Pretreatment	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Other (%)
Control	26.72±0.15	17.73±0.13	41.19±0.02	14.36±0.23
NaOH	41.81±3.44	20.35±0.59	25.49±0.71	12.36±3.32
IL	21.34±0.86	25.24±0.15	44.06±1.65	9.36±1.98
NaOH+IL	41.97±3.66	19.67±0.26	27.69±1.88	10.68±2.49

Figure 3   Time course of enzymatic hydrolysis (60°C for 48 hours) of coconut coir dust pretreated with 1% NaOH (80°C, 16 hours), IL (120°C, 15 hours), NaOH+IL and native coconut coir dust as control.

The compositions of cellulose, hemicellulose, and lignin in coconut coir dust were analyzed using the method recommended by Datta and Rathin [17]. The chemical composition of coconut coir dust after pretreatment and without pretreatment is shown in Table 1. The greatest lignin removal was found for coconut coir dust pretreated by NaOH and NaOH+IL. In contrast, pretreatment of IL showed the least lignin removal. The calculation of the entire coconut coir composition has been performed by other authors with different results [26].

3.3   Enzymatic Hydrolysis of Coconut Coir Dust Lignocellulose

The enzyme preparation and calculation of standard curves for glucose, xylose and total reducing sugar followed and adapted the standard method [27].

To investigate the effects of the various pretreatments on the enzymatic hydrolysis of the substrate, the enzymatic hydrolysis of non-pretreated and pretreated coconut coir dust were compared as shown in Figure 3. The TRS concentration was only 2.30 g/L for the non-pretreated substrate after 48 hours of enzymatic hydrolysis. After NaOH pretreatment, the TRS value was 4.05 g/L. The increase in the substrate digestibility of NaOH-pretreated coconut coir dust was attributed to the delignification of some lignin and the dissolution of hemicellulose. The cellulose was more open but still bound to lignin and hemicellulose so the enzymes easily attacked the cellulose. The TRS concentration of IL-pretreated coconut coir dust was 3.55 g/L.   To increase the performance of the enzymatic hydrolysis of coconut coir dust, NaOH pretreatment was followed by IL pretreatment (NaOH+IL). The TRS concentration of NaOH+IL-pretreated substrates was 4.46 g/L after 48 hours, which was the highest TRS concentration achieved by the different pretreatments. This result indicated that the TRS concentration could be significantly influenced by the synergy of the delignification and dissolution of hemicellulose, the openness of the cellulose surface and transformation of the crystal structure from cellulose I to cellulose II. This result was similar to others reported previously [28].

$$Yield = \frac{mass\ of\ TRS}{mass\ of\ dried\ substrate\ coconut\ coir\ dust, CCD} \qquad (1)$$

$$Yield = \frac{mass\ of\ TRS}{mass\ of\ theoritical\ TRS\ of\ native\ coconut\ coir\ dust\ (T\ TRS)} \qquad (2)$$

The mass of TRS was calculated by multiplying the TRS concentration by the suspension volume. T TRS is the theoretical total reducing sugars obtained from the chemical composition (cellulose+hemicellulose) of native coconut coir dust. For native coconut coir dust, T TRS was 0.267 multiplied by the mass of the dried CCD plus 0.177 multiplied by the mass of dried CCD.

Table 2 shows a comparative summary of the TRS concentration and HPLC measurement (glucose, xylose, and galactose) obtained from enzymatic hydrolysis by cellulase enzymes under various pretreatments at 60°C for 12 and 48 hours. In this study, the largest yield obtained was 0.13 g TRS/g dried coconut coir dust from the substrate that was pretreated with NaOH+IL. The hydrolysis yields of substrates pretreated by NaOH, IL and non-pretreated were 0.12, 0.11 and 0.07, respectively.

Table 2 Yield (Eq. (1)) and HPLC measurement of the enzymatic hydrolysis of substrates obtained by the various pretreatments for 12 and 48 hours.

| Methods | Concentration of reducing sugars after 12 and 48 h (HPLC) | | | | | | | | DNS | | Yield DNS | |
| | Glucose | | Xylose | | Galactose | | HPLC | | | | | |
	12 h	48 h	12 h	48 h	12 h	48 h	12 h	48 h	12 h	48 h	12 h	48 h
Control	0.83	0.83	0.01	0.02	0.04	0.04	0.88	0.88	2.30	2.30	0.07	0.07
NaOH	1.67	1.56	0.02	0.11	0.05	0.64	1.75	2.31	3.32	4.05	0.10	0.12
IL	1.57	1.26	0.00	0.00	0.03	0.38	1.60	1.64	2.87	3.55	0.09	0.11
NaOH+IL	1.93	1.32	0.06	0.12	0.06	0.62	2.05	2.06	3.48	4.46	0.10	0.13

Using Eq. (2), the highest yield was 0.30 for NaOH+IL-pretreated coconut coir dust, as shown in Table 3. Therefore, the NaOH+IL-pretreated substrate is the most easily hydrolyzed by water of all substrates. The compositions of glucose, xylose and galactose were measured by HPLC 10 times.

Generally, the yields of enzymatic hydrolysis of pretreated substrates were higher than those from the non-pretreated substrate. After pretreatment, the surface area of the cellulose increased, and the value of the crystalline index, CrI, decreased [28]. IL pretreatment damaged the crystalline structure of the cellulose in coconut coir dust so the enzymes could easily attack the cellulose. In the pretreated substrates, the atoms of the cellulose moved away from each other, resulting in an amorphous form.

Table 3 Comparison of yields of enzymatic hydrolysis of substrates obtained by various pretreatments for 12 and 48 hours using Eq. (1) and Eq. (2).

| Pretreatment | TRS concentration (g/L) | | Yield Eq.1 | | Yield Eq. 2 | |
	12 h	48 h	12 h	48 h	12 h	48 h
Control	2.30	2.30	0.07	0.07	0.16	0.16
NaOH	3.32	4.05	0.10	0.12	0.22	0.27
IL	2.87	3.55	0.09	0.11	0.19	0.24
NaOH+IL	3.48	4.46	0.10	0.13	0.23	0.30

The yield of enzymatic hydrolysis of IL-pretreated wheat straw was higher than that of non-pretreated wheat straw [29]. The yield of the enzymatic hydrolysis of NaOH-pretreated coconut coir dust was greater than the yield of the IL-

pretreated substrate. NaOH dissolved some lignin and hemicellulose so that the cellulose was exposed, providing the enzymes easy access to the cellulose and hemicellulose in coconut coir dust and enabling their conversion to fermentable sugars. In a study with poplar wood, the yield of enzymatic hydrolysis of IL-pretreated substrate was larger than that of NaOH-pretreated substrate because of the difference in lignin composition. The lignin content in coconut coir dust was 41.2%, while that of poplar wood was only 23% [30].

The NaOH+IL-pretreated substrate gave the highest yield of all the pretreatments because NaOH dissolved some of lignin so that IL could interact with the cellulose. The abundance of soluble cellulose in amorphous form made it easier for cellulose to decompose the substrate into sugars, as reported by other authors [29].

4   Conclusion

Reducing sugars have been successfully prepared from high-lignin lignocellulose from coconut coir dust pretreated by ionic liquid, alkaline solution and the combination of alkaline and ionic liquid. A comparative study was conducted to analyze the sugar yields from hydrolysis of the IL-, NaOH- and combined NaOH+IL-pretreated substrates, as well as a non-pretreated substrate as a control. The highest yield of sugar was 0.30g sugar/g cellulose+hemicellulose or 0.13 g sugar/g dried lignocelluloses for NaOH+IL-pretreated coconut coir dust. The yield of sugar from the non-pretreated substrate was 0.15. For coconut coir dust pretreated with IL or NaOH, the yields of reducing sugars were 0.24 and 0.27g sugar/g cellulose+hemicellulose, respectively.

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