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Reconstitution of Cellulose and Lignin After [C_2mim][OAc] Pretreatment and Its Relation to Enzymatic Hydrolysis

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ABSTRACT: Although the effects of cellulose crystallinity and lignin content as two major structural features on enzymatic hydrolysis have been extensively studied, debates regarding their effects still exist. In this study, reconstitution of cellulose and lignin after 1-ethyl-3-methylimidazolium acetate ([C_2mim][OAc]) pretreatment was proposed as a new method to study their effects on enzymatic digestibility. Different mechanisms of lignin content for reduction of cellulose hydrolysis were found between the proposed method and the traditional method (mixing of cellulose and lignin). The results indicated that a slight change of the crystallinity of the reconstituted materials may play a minor role in the change of enzyme efficiency. In addition, the present study suggested that the lignin content does not significantly affect the digestibility of cellulose, whereas the conversion of cellulose fibers from the cellulose I to the cellulose II crystal phase plays an important role when an ionic liquid pretreatment of biomass was conducted.

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KEYWORDS: cellulose; lignin; enzymatic hydrolysis; ionic liquid; pretreatment

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Introduction

Diminishing petroleum reserves and growing concerns about global climate change necessitate the development of fuel production pathways based on renewable resources (Kunkes et al., 2008). First-generation biofuels, fermented or refined from foodstuffs such as corn (maize), sugarcane, and soybeans, have contributed to high food prices and deforestation (Fairley, 2011). Therefore, second-generation biofuels appear to be on the cusp of research and development as well as commercialization (Fairley, 2011; Somma et al., 2010). Second-generation biofuels are derived from nonfood-based renewable resources, called lignocellulosic biomass, this includes agricultural and forestry residues, municipal waste, herbaceous, hardwood, and softwood crops.

Lignocellulosic biomass is composed of three major components: semicrystalline cellulose, a linear polymer of cellobiose (35–50%); hemicelluloses, a complex branched polymer of xylose and other sugar derivatives (20–35%); and lignin, a polyphenyl propanoid macromolecular assembly (5–30%) that is covalently cross-linked to hemicelluloses (Huber et al., 2006; Samayam et al., 2011). Several natural factors are believed to contribute to the recalcitrance of lignocellulosic biomass to chemicals and enzymes. These include the degree of lignification, the structural heterogeneity and complexity of cell–wall constituents, and the inhibitors to subsequent fermentations that exist naturally in cell walls or are generated during conversion processes (Cosgrove, 2005; Himmel et al., 2007; Iiyama et al., 1994; Wyman et al., 2005). The biomass recalcitrance must be overcome to explore biofuels from these renewable resources efficiently and economically. Besides researches focus on engineering plants and enzymes for biofuels production (Abramson et al., 2010; Sticklen, 2006), more attention has been paid to pretreatment of biomass. These pretreatment technologies can be classified into biological, physical, chemical, and physico-chemical methods. The

differences and advantages of each pretreatment method have been previously discussed and reviewed (Alvira et al., 2010; Hendriks and Zeeman, 2009; Himmel et al., 2007).

Among the various structural features affecting biomass enzymatic digestibility, cellulose crystallinity, and lignin content are considered to be two of the major substrate properties that influence saccharification kinetics and yields of enzymatic hydrolysis (Nishiyama et al., 2002; Zhu et al., 2008). In the native state, cellulose exists as a semicrystalline polymer, called cellulose I (and referring collectively to two naturally occurring allomorphs, Iα and Iβ), can be a major impediment to its hydrolysis to monomeric sugars (Samayam et al., 2011). The lignin composition as well as lignin content (Lee et al., 2009; Studer et al., 2011) has been considered to affect the enzymatic hydrolysis of cellulose. The composition of the functional groups of lignin (Kumar et al., 2011; Nakagame et al., 2011) and physical distribution such as syringyl (S) unit to guaiacyl (G) unit ratio (Li et al., 2010a,b; Studer et al., 2011) has also been demonstrated to affect the enzymatic hydrolysis of cellulose. Lignin appears to limit cellulose hydrolysis by two distinct mechanisms: firstly by forming a physical barrier that prevents enzyme access and secondly by non-productive binding cellulolytic enzymes (Pan et al., 2005). To reveal the underlying relationship between these two major structural features and the cellulose digestibility, it is important to investigate the influence of each one on the rate and extent of hydrolysis (Zhu et al., 2008).

Previous research has confirmed that decreasing the crystalline of cellulose and the content of lignin in the substrate significantly increased the enzymatic hydrolysis of cellulose (Abramson et al., 2010; Chundawat et al., 2011; Sticklen, 2006). However, it should be noted that most of the previous researches were conducted with pretreated biomass. Altering one structural feature often results in substantial changes in others. Therefore, it will confound us while understanding the relative importance of the studied features (Zhu et al., 2008). In order to overcome this obstacle a traditionally, microcrystalline cellulose (Avicel) and different lignin preparations were added to the enzymatic hydrolysis system.

A recent study used the ionic liquid 1-ethyl-3-methylimidazolium acetate ([C_2mim][OAc]) to pretreat poplar, switchgrass, and corn stover at 120°C for 1 h. The enzymatic hydrolysis results showed that the ionic liquid pretreatment resulted in nearly complete conversion of polysaccharides to monomeric sugars under high lignin contents (~19% to ~25%; Samayam et al., 2011). Moreover, to evaluate the interference of lignin in the saccharification of cellulose, Avicel was mixed with different amounts of yellow poplar lignin and pretreated with ionic liquid prior to enzymatic hydrolysis. The data did not show any significant difference in conversion efficiency between Avicel alone and Avicel with different amounts of lignin (Wang et al., 2011). These conflicting results further promote our doubt about the above-mentioned methods (mixing of cellulose and lignin) used to study the effect of lignin content on the enzymatic hydrolysis when an ionic liquid pretreatment of biomass was conducted. The more recent discoveries of dissolution of lignocellulosic biomass in ionic liquids, demonstrate at least partial separation of the major constituent biopolymers, promoting ionic liquid pretreatment as a potential alternative to established pretreatment techniques (Mora-Pale et al., 2011; Ouellet et al., 2011; Stark, 2011; Sun et al., 2011; Tadesse and Luque, 2011).

In this work, a new method was proposed to study the effects of cellulose crystallinity and lignin content on enzymatic hydrolysis of cellulose when an ionic liquid pretreatment of biomass was conducted. The ionic liquid [C_2mim][OAc] has been used by previous studies in order to separate lignin from the lignocellulosic biomass (Dibble et al., 2011; Kim et al., 2011; Sun et al., 2009). In this study, cellulose and lignin were reconstituted as a function of [C_2mim][OAc]. Microcrystalline cellulose and milled wood lignin (MWL) were used as model substrates. Both microcrystalline cellulose and MWL have been considered to be representative source of pure cellulose and native lignin. The characterization of the reconstituted material and the major features which affect the enzymatic hydrolysis were investigated.

Materials and Methods

Materials

The ionic liquid [C_2mim][OAc] (≥98.5%) was purchased from Lanzhou Institute of Chemical Physics, Lanzhou, China. Microcrystalline cellulose (DP: 210–240) was purchased from Sinopharm Chemical Reagent Co., Ltd. The MWL was isolated and purified from ball-milled triploid of *P. tomentosa* Carr. meal according to a previous literature (Yuan et al., 2011). The raw material used was a fast-growing popular tree, 6 years old, which was harvested from Shandong province, China. The Klason lignin content was measured by the TAPPI standard, which amounts to 19.9% of the dry wood. Commercial cellulase preparation (Celluclast 1.5 L) and β-glucosidase, produced by *Tricoderma reesei* ATCC 26921 and almonds, respectively, were purchased from Sigma-Aldrich. All other chemicals used were of analytical or reagent grade and directly used as purchased without further purification.

Reconstitution of Cellulose and Lignin

To ensure the complete dissolution of cellulose and lignin, 600 mg microcrystalline cellulose and a chosen amount of MWL (0, 100, 200, and 300 mg) was added to 20 g of [C_2mim][OAc] in a 50 mL dried three-neck flask. The mixture was then placed into an oil bath and heated on a hot plate (IKA RCT basic, Germany) with vigorous magnetic stirring (600 rpm) at 90°C for 3 h under an N_2 atmosphere. After the complete dissolution of cellulose and lignin, the

reconstitution of them was carried out by precipitation of the heated solution into 400 mL hot deionized water (~70°C) under vigorous magnetic stirring for 1 h. The reconstituted material was obtained by filtering through a cellulose nitrate membrane filter (pore size 0.45 μm, Whatman®), thoroughly washing with deionized water, and freeze-drying.

To investigate the physical structure of MWL after the ionic liquid dissolution and regeneration processes, 150 mg MWL was dissolved in 20 g of [C_2mim][OAc] under the same conditions. The regeneration of MWL was carried out by precipitation of the heated solution into 400 mL acidic deionized water (pH = 2) under vigorous magnetic stirring for 1 h. The regenerated MWL (RMWL) was obtained by filtering through the cellulose nitrate membrane filter, thoroughly washing with acidic deionized water (pH = 2), and freeze-drying.

Composition of Reconstituted Material

The cellulose and lignin contents in the reconstituted material were determined in duplicate using concentrated acid hydrolysis followed by dilute acid hydrolysis according to the standard laboratory analytical procedures developed by the National Renewable Energy Laboratory (NREL) (Sluiter et al., 2008). The total lignin content was the summation of acid-soluble lignin and acid-insoluble lignin. The glucose was quantified by high-performance anion-exchange chromatography (HPAEC) system (Dionex ICS3000, Sunnyvale, CA) with a pulsed amperometric detector, a AS50 autosampler, a Carbopac™ PA-20 column (4 × 250 mm, Dionex), and a guard PA-20 column (3 × 30 mm, Dionex) according to a previous literature (Yuan et al., 2010). The cellulose content was calculated based on glucose using anhydro corrections of 0.9.

Enzymatic Hydrolysis

Enzymatic hydrolysis of all samples was performed in 50-mL Erlenmeyer flasks at 50°C in a shaking air bath at 150 rpm for 36 h. A typical hydrolysis mixture consisted of 0.1 g of sample, 10 mL of 50 mM sodium acetate buffer (pH 4.8) supplemented with 40 μL antibiotics tetracycline and 20 μL cycloheximide, and 35 FPU/g substrate of cellulase and 37.5 IU/g substrate of β-glucosidase. After hydrolysis, 100 μL samples was taken from the reaction mixture and centrifuged for 10 min at 10,000 rpm. The released glucose was also analyzed by HPAEC (Dionex, ISC3000) but equipped with a CarboPac PA 100 analytical column. All experiments were carried out in duplicate.

Characterization

The structural features of MWL were characterized according to a previous literature (Yuan et al., 2011). The carbohydrate moieties associated with MWL were determined by hydrolysis with dilute sulfuric acid. The weight-average (M_w) and number-average (M_n) molecular weights of MWL were determined by gel permeation chromatography (GPC) on a PL-gel 10 mm Mixed-B 7.5 mm i.d. column. The syringyl (**S**) unit to guaiacyl (**G**) unit ratio was calculated from the two-dimensional heteronuclear single-quantum coherence (2D HSQC) spectrum of MWL.

X-ray powder diffraction patterns of the microcrystalline cellulose and the reconstituted materials were obtained using an XRD-6000 instrument (Shimadzu, Japan). The X-ray diffractograms were recorded from $2\theta = 5$ to 35° using reflection method at a scanning speed of 2°/min. The determination was performed with Ni-filtered Cu-K_α radiation ($\lambda = 1.54$ Å) at 40 kV and 40 mA. The crystallinity of the samples was quantified from X-ray powder diffraction data using a crystallinity index, CrI, for cellulose (Segal et al., 1959).

Changes in morphology of cellulose and lignin before and after ionic liquid reconstitution and enzymatic hydrolysis were observed by scanning electron microscopy (SEM). For magnification, an S-3400N (HITACHI, Tokyo, Japan) scanning electron microscope was operated at 10 kV acceleration voltages. Prior to imaging, samples were coated with gold-palladium by a sputter coater (E-1010, HITACHI).

Results and Discussion

Characterization of MWL

As previously mentioned in the introduction, the structural features of lignin as well as the amount of lignin are believed to be largely responsible for the negative effects of lignin on hydrolysis (Kumar and Wyman, 2009). Therefore, in the present study, some structural features of MWL were characterized according to a previous literature (Yuan et al., 2011). The yields of MWL and the carbohydrate moieties associated with MWL were 16.6% and 3.18%, respectively. MWL contained a larger percentage of xylose (67.6%) among the total sugars and uronic acids. Rhamnose, mannose, and glucose appeared to be the secondary major sugars, comprising 6.3%, 5.7%, and 4.4% of the total sugars and uronic acids, respectively. Arabinose and galactose were observed as noticeable amounts. The weight-average (M_w) molecular weight, calculated from the GPC curve (relative value related to polystyrene), of MWL was 2,610 g/mol. The polydispersity (M_w/M_n) of MWL was 2.16. The **S/G** ratio, which was calculated from the 2D HSQC spectrum of MWL (not shown), was estimated to be 1.65. It should be mentioned that the different results obtained herein as compared to the previous study can be attributed to the different raw material used, although the MWL was isolated and purified with the same method.

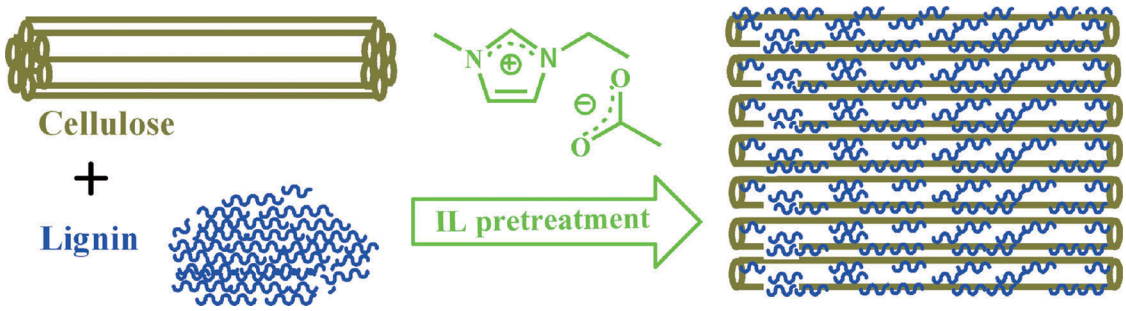

Figure 1. Schematic representation of the reconstitution of cellulose and lignin in the ionic liquid [C_2mim]OAc.

Compositional Analysis

More recently, room temperature ionic liquids have been used as solvents for lignocellulosic biomass processing with the aim of developing alternatives for lignocellulosic pretreatment (Mora-Pale et al., 2011). For example, previous studies have attempted to separate lignin from lignocellulosic biomass with [C_2mim][OAc] (Dibble et al., 2011; Kim et al., 2011; Sun et al., 2009). Conversely in the present study, microcrystalline cellulose and MWL were reconstituted as a function of [C_2mim][OAc] pretreatment as described in Figure 1. It has been confirmed that not all the dissolved components would be regenerated from the ionic liquid system by adding an anti-solvent. Therefore, the chemical components of the reconstituted materials were determined (Table I). The results showed that the lignin contents of the samples 3, 4, and 5 were 12.8%, 22.8%, and 32.6%, respectively. These figures indicate that only a small amount of lignin was lost during the reconstitution process. This was in line with the results of Samayam et al. (2011) who studied the weight percent of lignin in native and [C_2mim][OAc] treated (at 50 and 120°C) poplar, switchgrass, and corn stover. The modification of the structural features of MWL during the reconstitution process for the samples 3, 4, and 5 were presumed to be similar and limited in the present study. Kim et al. (2011) showed that the amounts of functional groups appeared to be similar for MWL and the lignin extracted with [C_2mim][OAc] from a poplar wood even the pretreatment was carried out at 110°C for 16 h.

Table I. Chemical components of samples prepared by different methods.

		Content (%)	
Prepared method	Sample	Lignin	Cellulose
Microcrystalline cellulose	1	0	100
IL regenerated microcrystalline cellulose	2	0	100
Reconstitution of microcrystalline cellulose and lignin after IL pretreatment	3	12.8	87.2
	4	22.8	77.2
	5	32.6	67.4
Mixing of IL regenerated microcrystalline cellulose and lignin	6	14.3	85.7
	7	25.0	75.0
	8	33.3	66.7

Enzymatic Hydrolysis

The enzymatic hydrolysis of microcrystalline cellulose and various reconstituted materials were conducted in order to evaluate the effects of cellulose crystallinity and lignin content on enzymatic hydrolysis of cellulose. The conversion of cellulose to glucose at 36 h is given in Figure 2. After 36 h, 98.6% of the regenerated microcrystalline cellulose was converted to glucose compared with 51.6% of the raw microcrystalline cellulose. The significant increase of cellulose digestibility of ionic liquid pretreated cellulose was due to the increase of the surface area (Dadi et al., 2007; Silva et al., 2011) and the conversion of cellulose fibers from the cellulose I to the cellulose II crystal phase (Samayam

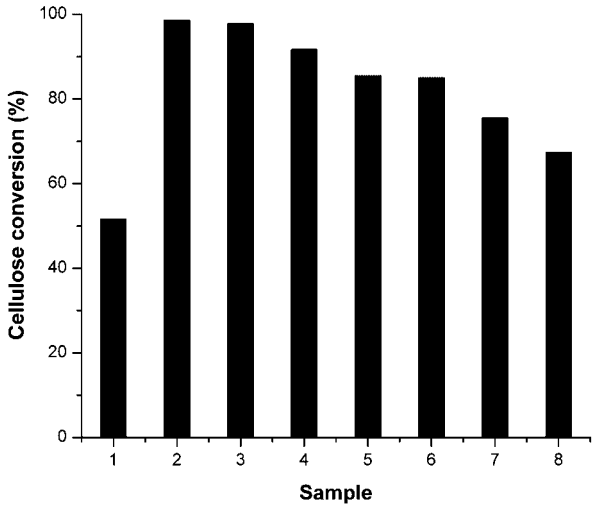

Figure 2. Thirty-six hour cellulose conversion of various samples.

et al., 2011). This view was confirmed by the following XRD and SEM analyses. For samples 3, 4, and 5, the yields of glucose were 97.8, 91.7, and 85.5%, respectively. As shown in Table I, the lignin content of the samples 3, 4, and 5 was 12.8, 22.8, and 32.6%, respectively. These results indicated that even at high lignin content the 36 h yield of glucose for the reconstituted materials was over 85% in the present study.

Our recent studies have found that the cellulose digestibility of the [C_2mim]OAc/alkali pretreated poplar wood reached 94.9% after 24 h and 99.2% within 48 h although with a relatively high content of lignin (15.1%) (unpublished data). In addition, Samayam et al. (2011) also confirmed that the [C_2mim]OAc pretreated biomass resulted in nearly complete conversion of polysaccharides to monomeric sugars under high lignin contents (~19% to ~25%). All these results suggested that the lignin content does not significantly affect the digestibility of cellulose when an ionic liquid pretreatment of biomass was conducted. It should be mentioned that the high enzyme dosage applied might mask the observations and phenomena related to the interactions between cellulose and lignin. The enzymatic hydrolysis of the reconstituted materials with a lower enzyme dosage should be carried out in the future.

The 36 h yields of glucose declined dramatically to 85.0–67.4% of samples 6, 7, and 8. These samples were composed of regenerated microcrystalline cellulose mixed with different amounts of MWL. However, it should be noted that the lignin contents of the samples 6, 7, and 8 were only slightly higher than those of the samples 3, 4, and 5. These obvious differences in the cellulose digestibility between the reconstituted materials (samples 3, 4, and 5) and the substrates composed of regenerated microcrystalline cellulose mixed with different amounts of MWL (samples 6, 7, and 8) may be attributed to the different mechanisms of digestion inhibition upon increasing lignin content.

Effect of Lignin Content on Digestibility

The 36 h yield of glucose of both types of substrates, samples 3–5 and samples 6–8, as a function of lignin content is exhibited in Figure 3. There was an apparently negative, linear correlation between the two variables, that is, the efficiency of enzymatic hydrolysis dropped as the lignin content of the substrate increased. However, not only the correlation coefficient but also the slope of the two lines was discrepant. For the dotted line, the correlation coefficient was 0.9998 whereas it was only 0.8812 for the solid line. In addition, the slope of the dotted line was clearly higher than that of the solid line. These results further confirmed that the mechanisms of lignin for reduction of cellulose hydrolysis for the two types of substrates were different. For the samples 6, 7, and 8, it seems that the efficiency of enzymatic hydrolysis just affected by the content of lignin ($R^2 = 0.9998$), which appears to reduce cellulose hydrolysis by non-productive binding cellulolytic enzymes. Pan et al. (2005) reported that in addition to the physical barrier

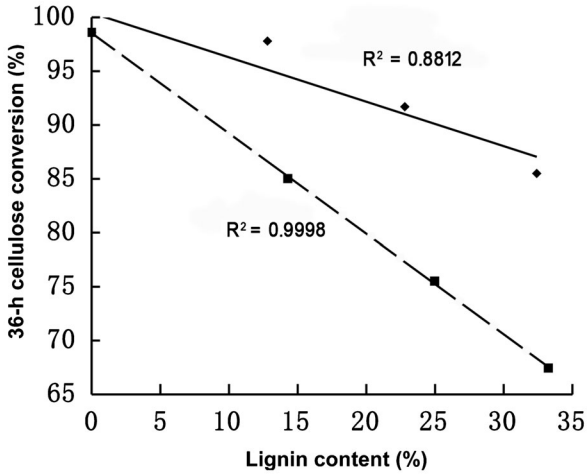

Figure 3. Correlation between lignin content and enzymatic digestibility of various samples. The solid line represented the samples 2, 3, 4, and 5 and the dotted line represented the samples 2, 6, 7, and 8.

imposed by the lignin matrix, enzyme–lignin interactions play a critical role in reducing enzyme efficiency. However, for the samples 3, 4, and 5, other effects on the enzymatic hydrolysis need to be considered in addition to the effect of the lignin content.

XRD Analysis

The structure of raw and regenerated microcrystalline cellulose as well as the reconstituted materials (samples 3–5) was examined by XRD in order to gain insight into the possible structural features affecting the cellulose hydrolysis. The CrI of the samples 1–5 was calculated to be 60.3, 45.0, 42.9, 42.5, and 40.7%, respectively. Representative Miller indices for the reflections (1–10), (110), and (200) for cellulose I and (1–10), (110), and (020) for cellulose II are labeled in Figure 4, although there are overlapping contributions from others (Samayam et al., 2011). After regeneration and reconstitution under the conditions in the present study, all the samples 2–5 have recrystallized in the cellulose II phase. The slight decrease of the CrI for the samples 2–5 was due to the gradual increase of amorphous lignin, although the corresponding digestibility of the substrates decreased. The hydrolysis of cellulose II was more complete than that of cellulose I (Cheng et al., 2011; Samayam et al., 2011; Wada et al., 2010), which was the reason for the high digestibility of the ionic liquid pretreated materials. It has been reported that for high-lignin and low-crystallinity poplar wood, the ultimate extent of hydrolysis may not be very high because lignin may block enzyme accessibility to some cellulose sites (Zhu et al., 2008). This was inconsistent with the present study, as even at high lignin content the 36 h yield of glucose for the reconstituted materials was over 85%. However, the structure of the

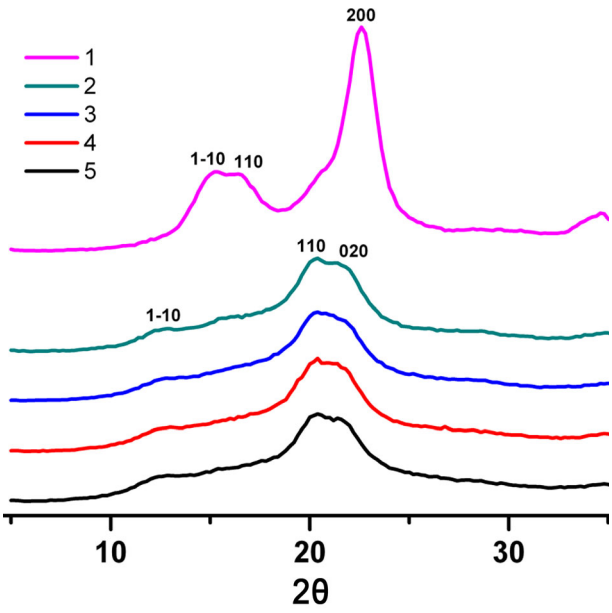

Figure 4. X-ray diffractograms of various samples.

reconstituted materials was far from biomass. Biomass is composed of three major components, cellulose, hemicelluloses, and lignin, which form an intact cell wall matrix. It is likely that a slight change of the crystallinity of the reconstituted materials may play a minor role in the change of enzyme efficiency. This deduction was supported by Park et al. (2010), who caution against trying to correlate relatively small changes in CrI with changes in cellulose digestibility.

SEM Analysis

SEM images of raw and regenerated microcrystalline cellulose, MWL, RMWL, reconstituted materials, and the residue of sample 5 after enzymatic hydrolysis (5-EH) were taken in order to investigate the morphology change caused by [C_2mim][OAc] reconstitution and enzymatic hydrolysis (Fig. 5). The untreated microcrystalline cellulose has a highly fibrillar and intact morphology. Conversely, the surface morphology of the ionic liquid-treated microcrystalline cellulose showed no fibrous structure and pores were observed. This was in accordance with the results obtained by Li et al. (2010) who studied the pretreatment of switchgrass with [C_2mim][OAc]. In addition, the surface area of the ionic liquid-pretreated cellulose was significantly increased, which has been confirmed by several recent

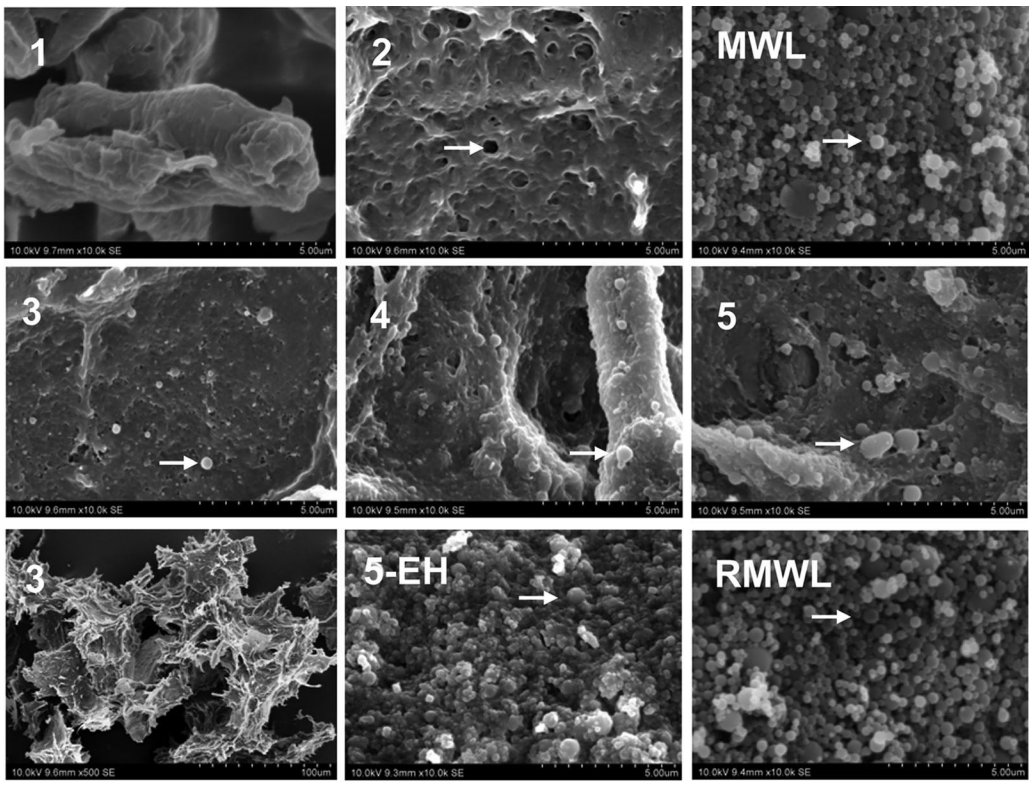

Figure 5. SEM photomicrographs of MWL, RMWL, and various samples.

studies (Dadi et al., 2007; Silva et al., 2011). The increase of the surface area and the decrease of the crystallinity resulted in the high enzymatic digestibility of the ionic liquid-pretreated cellulose. It can also be observed that the MWL and RMWL were nanoscopic particles over several diameter scales. The difference in the diameter of MWL before and after ionic liquid pretreatment was limited (Fig. 5, MWL and RMWL). After enzymatic hydrolysis, the SEM image of the residue of sample 5 (5-EH) at 10,000× magnification was also given in Figure 5. As shown in Figure 5 (5-5H), the lignin still forms droplets after enzymatic hydrolysis. However, whether the lignin separated from the cellulose and suspended itself in the buffer solution when the enzymatic hydrolysis was performed cannot be detected in the present study.

The surface morphology significantly changed with the adding of MWL for the reconstituted materials. The increasing amount of MWL in the reconstituted materials used in samples 3–5 resulted in a change in the size and number of pores, observed in the surface of the ionic liquid-treated microcrystalline cellulose (sample 2). As can been seen in Figure 5 (samples 3–5), the reconstituted materials were small particles. Much lignin embedded within the reconstituted materials and a little of lignin adhered to the surface of them. The MWL nanoscopic particles reconstituted with microcrystalline cellulose and diminished the pores as a function of ionic liquid pretreatment. In this case, the MWL embedded within the reconstituted materials may form a physical barrier, which prevented the accessibility of enzyme to the cellulose molecules. The decrease of the surface area may also play a critical role in reducing the digestibility of the reconstituted materials. The other important change was the presence of sphere droplets on the surface of the reconstituted materials. Similar droplets have also been found on the surface of dilute acid and AFEX pretreated biomass, which have been formed as the redistribution of lignin and the cell wall decomposition products, respectively (Chundawat et al., 2011; Sannigrahi et al., 2011; Selig et al., 2007). Clearly, the reconstitution conditions in the present study were milder than those during dilute acid and AFEX pretreatment processes. The operation temperature of ionic liquid reconstitution was just 90°C, lower than the glass-transition temperature of lignin (100–160°C) (Samayam et al., 2011). Furthermore, Singh et al. (2009) confirmed the rejection of lignin from the recovered polysaccharides via the addition of water to the ionic liquid-dissolved switchgrass. Therefore, the droplets adhered to the surface of the reconstituted materials were presumed to be MWL. These droplets may be detrimental to enzymatic hydrolysis due to the non-productive binding of enzymes to MWL (Sannigrahi et al., 2011).

Conclusions

Reconstitution of cellulose and lignin after $[C_2mim][OAc]$ pretreatment was proposed as a new method to study the effects of cellulose crystallinity and lignin content on enzymatic hydrolysis of cellulose. Different mechanisms of lignin content for reduction of cellulose hydrolysis were observed between the proposed method and the traditional method. In addition, the present study suggested that the lignin content does not significantly affect the digestibility of cellulose, whereas the conversion of cellulose fibers from the cellulose I to the cellulose II crystal phase plays an important role when an ionic liquid pretreatment of biomass was conducted.

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