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Ionic Liquid-Mediated Selective Extraction of Lignin From Wood Leading to Enhanced Enzymatic Cellulose Hydrolysis

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ABSTRACT: Lignocellulose represents a key sustainable source of biomass for transformation into biofuels and bio-based products. Unfortunately, lignocellulosic biomass is highly recalcitrant to biotransformation, both microbial and enzymatic, which limits its use and prevents economically viable conversion into value-added products. As a result, effective pretreatment strategies are necessary, which invariably involves high energy processing or results in the degradation of key components of lignocellulose. In this work, the ionic liquid, 1-ethyl-3-methylimidazolium acetate ([Emim][CH_3COO]), was used as a pretreatment solvent to extract lignin from wood flour. The cellulose in the pretreated wood flour becomes far less crystalline without undergoing solubilization. When 40% of the lignin was removed, the cellulose crystallinity index dropped below 45, resulting in >90% of the cellulose in wood flour to be hydrolyzed by *Trichoderma viride* cellulase. [Emim][CH_3COO] was easily reused, thereby resulting in a highly concentrated solution of chemically unmodified lignin, which may serve as a valuable source of a polyaromatic material as a value-added product.

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KEYWORDS: cellulose crystallinity; delignification; enzymatic digestibility; ionic liquid; lignocellulose

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Introduction

The rapidly growing demand for energy, a dwindling and unstable supply of petroleum, and the emergence of global warming by use of fossil fuels have rekindled a strong interest in pursuing alternative and renewable energy sources. Lignocellulosic biomass, such as agricultural residues, forestry wastes, waste paper, and energy crops, has long been recognized as a potential sustainable source of sugars for biotransformation into biofuels and value-added bio-based products (Himmel et al., 2007; Li et al., 2008). Conversion of lignocellulosic materials into biofuels, for example, typically includes three steps: (1) pretreatment of lignocellulose to enhance the enzymatic or microbial digestibility of polysaccharide components; (2) hydrolysis of cellulose and hemicellulose to fermentable reducing sugars; and (3) fermentation of the sugars to liquid fuels or other fermentative products (Galbe and Zacchi, 2007; Sun and Cheng, 2002; Zhang et al., 2007).

A major concern in lignocellulose conversion is overcoming biomass recalcitrance through pretreatment while still maintaining green and energy efficient processing. Lignocellulose is composed of three major biopolymers—cellulose, hemicellulose, and lignin—all of which have distinct chemical, physical, and structural differences. Hemicellulose is relatively amorphous and is readily degraded by glycosidases, primarily xylanases, to yield fermentable sugars. Cellulose, however, is highly crystalline in lignocellulose and that protects it from chemical and biological degradation. Lignin is a highly branched, aromatic polymer, composed of phenylpropanoid units

that serve as the "glue" that binds cellulose and hemicellulose, imparting rigidity, and moisture and microbial resistance to lignocellulose (Chandra et al., 2007). Due to the protective features of lignin, and its inability to undergo rapid biotransformation into value-added chemicals, it is not surprising that the vast majority of lignocellulosic pretreatment strategies have focused on achieving a reduction in biomass lignin content and at the same time attempting to achieve a reduction in cellulose crystallinity without destroying the fermentable sugar content of the lignocellulose.

Numerous approaches have been used to remove lignin. Examples include physical (e.g., limited pyrolysis and mechanical disruption/comminution, Mosier et al., 2005), physicochemical (e.g., steam explosion, ammonia fiber explosion, Grous et al., 1986; Mes-Hartree et al., 1988), chemical (e.g., acid hydrolysis, alkaline hydrolysis, high temperature organic solvent pretreatment, oxidative delignification, Chum et al., 1988; Gierer and Noren, 1982; Zhang et al., 2007), and biological (e.g., lignin degradation by white- and soft-rot fungi, Hatakka, 1983; Lee, 1997) methods. In all cases, upon sufficient removal of the lignin, there was also substantial degradation of lignin and in many cases there was substantial loss in fermentable sugar content of the residual polysaccharides (Galbe and Zacchi, 2007). The degradation of lignin is unfortunate, resulting in loss of a highly functional natural product that comprises 20–35% of the mass of lignocellulose. A key challenge, therefore, remains; namely, achieving the efficient and selective removal of near native lignin coupled with the enhanced biodegradability of cellulose and hemicellulose.

Ionic liquids (ILs) are organic salts that usually melt below 100°C. Interest in ILs stems from their potential application as "green solvents" (Sheldon et al., 2002). Specifically, due to their high thermal stability and nearly complete non-volatility, ILs are becoming attractive alternatives to volatile and unstable organic solvents. In chemical processes, ILs exhibit excellent physical characteristics including the ability to dissolve polar and non-polar organic, inorganic, and polymeric compounds (Lee and Lee, 2005). Moreover, there is almost a limitless combination of anions and cations that can be used to synthesize ILs (Freemantle, 1998).

ILs have been shown to be capable of dissolving cellulose (Zhu et al., 2006), which may have practical advantages for enzymatic hydrolysis of cellulose (Kamiya et al., 2008). For example, cellulose reconstituted after being dissolved in [Amim][Cl] and [Bmim][Cl] had lower degrees of crystallinity than native cellulose, which resulted in greater accessibility of the polysaccharide chains to cellulases, and thus more facile hydrolysis than was achieved without initial dissolution in and reconstitution from an IL (Dadi et al., 2006, 2007; Liu and Chen, 2006). More recently, several groups have reported the dissolution of full lignocellulosic materials such as corn stalks, rice straw, bagasse, pine wood, and spruce wood in ILs followed by cellulose hydrolysis with acid or enzymes (Fort et al., 2007; Kilpeläinen et al., 2007; Li et al., 2008).

In the current work, we have discovered a set of ILs that possess very high solubility for lignin with low solubility for cellulose and other constituents of wood flour. As a result, we were able to selectively *extract* lignin from lignocellulose and simultaneously yield a highly biodegradable cellulose fraction. Moreover, the extracted lignin is expected to be chemically unaltered, thereby providing an unadulterated source of raw material for use as a binder, dispersant, and emulsifier (Chakar and Ragauska, 2004), as well as potential as a blending agent in commodity plastics yielding enhanced composites (Kadla et al., 2002). The selective extraction of lignin from lignocellulosic biomass, therefore, may provide a route toward increased *total* utilization of lignocellulosic biomass.

Materials and Methods

Reagents

Cellulase from *Trichoderma viride*, citric acid, sodium citrate, 3,5-dinitrosalicylic acid (DNS), sodium hydroxide, sodium potassium tartarate, phenol, sulfuric acid, and all ILs were obtained from Sigma–Aldrich (St. Louis, MO). Indulin AT, a purified softwood kraft lignin from pine, was provided by MeadWestvaco (Charleston, SC). Maple wood flour (particle size = 250 μm) was graciously donated by P. J. Murphy Forest Products (Montville, NJ).

Solubilities of Lignin and Wood Flour in ILs

Kraft lignin (Indulin AT, 50 mg) was added to glass vials containing [Bmim][BF_4] or [Bmim][PF_6] (1 g) to measure the solubility of lignin in ILs. The resulting suspension was stirred for 24 h at 90°C. After centrifugation for 1 min at 14,000 rpm, the supernatant (0.1 g) was removed and diluted with 0.9 mL of 0.1 N NaOH to determine the total dissolved lignin content. The content of lignin was measured on a UV-Vis spectrophotometer (UV 1240; Shimadzu, Columbia, MD) at 280 nm with Indulin AT as the standard. For ILs ([Mmim][$MeSO_4$], [Bmim][CF_3SO_3], [Emim][CH_3COO], [Amim][Cl], [Bmim][Cl], and [Bzmim][Cl]) that were particularly good solvents for lignin dissolution, 0.5 g of lignin was added to the IL (5 g) with mechanical stirring until dissolved. Then an additional 0.5 g lignin was introduced and the solution was stirred at 90°C until it became homogeneous. To measure the solubility of wood flour in ILs, 50 mg wood flour was added to 10 g IL with mechanical stirring under a N_2 atmosphere. Additional wood flour (50 mg) was then added and stirred for 24 h at 80°C until the wood flour particulates disappeared.

Extraction of Lignin From Wood Flour

A 50 g/kg solution of maple wood flour was incubated in various ILs at 80°C under N_2 with magnetic stirring. After 24 h, the ILs solutions were diluted with 0.1 N NaOH, the suspension was centrifuged at 14,000 rpm for 5 min, and the supernatants removed and analyzed for lignin content by measuring absorbance at 280 nm. To determine the total lignin content of untreated wood flour, 0.1 g wood flour was dissolved in 10 g [Bmim][Cl] for 24 h at 80°C. The fully dissolved wood flour solution was diluted with 0.1 N NaOH, centrifuged, and the lignin content of the supernatant was obtained from its absorbance at 280 nm. The lignin content for untreated maple wood flour was 21.4%, and this served as the lignin content in untreated wood flour.

Pretreatment of Wood Flour With ILs

Maple wood flour was prepared in [Emim][CH_3COO] at a concentration of 50 g/kg. After incubation at various temperatures under magnetic stirring for a fixed time, 0.1 g of the suspension was removed to measure extracted lignin content (as described above). The remaining wood flour suspension was diluted with deionized water (at 10-fold higher mass than the IL) under stirring and the suspended wood flour was recovered by filtration. The recovered wood flour was washed with ethanol and dried in an oven at 80°C for 1 h. The mass of recovered wood flour was then determined.

Compositional Analysis of Wood Flour

Cellulose and xylan contents of all samples were determined by quantitative saccharification upon acid hydrolysis and subsequent HPLC analysis, based on the standard NREL procedure No. 002 (NREL, 1996). The sample was treated with 72% (v/v) sulfuric acid at 30°C for 2 h, followed by dilute acid (4%) at 121°C for 1 h. The hydrolysis products (glucose and xylose) were determined by HPLC (Shimadzu Model LC-10A*i*) equipped with a RI detector and a Bio-Rad HPX-87P column operated at 85°C. The mobile phase consisted of deionized water with a flow rate of 0.6 mL/min. The cellulose and xylan contents were calculated from glucose and xylose contents multiplied by conversion factors of 0.90 and 0.88, respectively (Zhu et al., 2008). The acid-insoluble lignin after acid hydrolysis was measured as the mass of insoluble residue remaining at 575°C. The acid-soluble lignin was measured by UV-Vis spectrophotometer at 205 nm with an extinction coefficient value of 110 L/g cm (NREL, 1996).

Enzymatic Hydrolysis

Enzymatic (cellulase-catalyzed) hydrolysis reactions were performed in 20-mL vials on a rotary shaker at 200 rpm and 37°C in volumes of 3.5 mL with a cellulase concentration of 34 U/mL in 50 mM citrate buffer (pH 4.7). Samples (50 μL) were periodically removed and boiled for 3 min to quench the enzymatic reaction. After centrifugation of the boiled samples, the total reducing sugar concentration of the supernatants was measured by the DNS assay against a glucose standard (Miller, 1959). Glucose concentration was also measured by HPLC, as described above. All reactions were performed in triplicate.

Cellulose Crystallinity Measurement

Pretreated and untreated wood flour were analyzed by X-ray diffraction. The samples were scanned on a D8 Discovery Diffractometer (Brüker, Madison WI) from $2\theta = 8$–30° with a scan speed of 0.1°/min and a step size of 0.01°. The cellulose crystallinity index (CrI) was determined via Equation (1) (Thygesen et al., 2005).

$$\mathrm{CrI} = \frac{I_{\mathrm{tot}} - I_{\mathrm{am}}}{I_{\mathrm{tot}}} \times 100 \qquad (1)$$

I_{am} is the integration of kraft lignin intensity and I_{tot} is the integration of sample intensity. The latter is assumed to be the sum of contributions from crystalline and amorphous wood flour fractions.

Results and Discussion

Critical challenges in designing a pretreatment strategy for enhancing the yield of polysaccharides from lignocellulosic biomass include achieving a high degree of lignin removal without destroying the fermentable sugar content of the biomass, increasing the degradability of cellulose and hemicellulose, and retaining the structural and chemical features of the lignin for further potential use. Selective extraction of lignin under mild conditions and without chemical conversion, while reducing the crystallinity of the cellulose, represents an ideal strategy to achieve improved biomass bioconversion, and is the central focus of this work. Thus, we initially set out to identify ILs that could dissolve high concentrations of lignin, would not dissolve wood flour, yet would result in a substantial decrease in the crystallinity of the cellulose.

Pretreatment of Wood Flour in Various ILs

The solubilities of kraft lignin and wood flour in ILs are shown in Table I. Imidazolium-based cations possess a wide range of lignin and wood flour solubilizing capacity depending on the associated anion (Pu et al., 2007). The highest lignin solubility was obtained using [Mmim][$MeSO_4$] and [Bmim][CF_3SO_3]; solvents that do not result in appreciable solubility of wood flour. At the other end of the spectrum, $[Cl]^-$-containing anions enabled relatively high wood flour solubility (10–30 g/kg) while retaining

Table I. Solubilities and extraction efficiency of lignin in various ionic liquids.

ILs	Hildebrand solubility parameter (δ_H) of ILs[a]	Hydrogen bond basicity (β) of ILs[b]	Lignin solubility (g/kg)[c]	Wood flour solubility (g/kg)[d]	Extracted lignin content (g/kg)[e]
[Mmim][$MeSO_4$]	ND[f]	ND	>500	ND	0.8
[Bmim][CF_3SO_3]	24.9	0.46	>500	ND	0.5
[Emim][CH_3COO]	ND	ND	>300	<5	4.4
[Amim][Cl]	ND	0.83	>300	>30	5.2
[Bmim][Cl]	ND	0.84	>100	>30	3.2
[Bzmim][Cl]	ND	ND	>100	>10	1.9
[Bmim][BF_4]	31.6	0.38	40	ND	ND
[Bmim][PF_6]	30.2	0.21	~1	ND	ND

[a]Hildebrand solubility parameter values from Swiderski et al. (2004).
[b]Kamlet-Taft hydrogen bond basicity values from Crowhurst et al. (2003) and Fukaya et al. (2006).
[c]Solubility of Indulin AT (kraft lignin) at 90°C after 24 h incubation.
[d]Solubility of wood flour after 24 h incubation at 80°C under N_2. ND indicates <1 g/kg.
[e]0.5 g wood flour was incubated in 10 g ILs for 24 h at 80°C under N_2. Lignin content was determined with the Indulin AT standard. ND indicates <0.1 g/kg.
[f]No data available.

>100 g/kg lignin solubility. [Bmim][BF_4] and [Bmim][PF_6] were not effective at dissolving either lignin or wood flour.

Lignin solubilities can be rationalized by examining their Hildebrand solubility parameters (Table I). The Hildebrand solubility parameter (δ_H) has been widely used for predicting the solubilities of various polymers in solvents. Maximum solubility is observed when the δ_H values of the polymer and solvent are identical, since the solubility of two materials is facilitated when their intermolecular attractive forces are similar (Lee and Lee, 2005). Thus, the high solubility of lignin in [Bmim][CF_3SO_3] is not surprising given the very close similarity of the δ_H values for both the IL and lignin (24.9 and 24.6, respectively). Conversely, the δ_H values for [Bmim][PF_6] and [Bmim][BF_4] are 30.2 and 31.6, respectively (Swiderski et al., 2004; Thielemans and Wool, 2005), which are sufficiently distinct from that of lignin, and hence do not favor high lignin solubility.

The high solubility of wood flour in $[Cl]^-$-containing ILs is consistent with reports in the literature (Fort et al., 2007; Kilpeläinen et al., 2007; Li et al., 2008). This may be rationalized by the high solubility of the predominant wood constituent, cellulose, in such solvents. Recent NMR studies on the dissolution mechanism of cellulose in [Bmim][Cl] indicate that $[Cl]^-$ acts as a hydrogen bond acceptor, which interacts with the hydroxyl groups of cellulose (Moulthrop et al., 2005). A good correlation of β values, corresponding to the hydrogen bond accepting capacity of ILs, with cellulose dissolution has been reported (Fukaya et al., 2006, 2008). It is not surprising, then, that $[Cl]^-$-containing ILs showed the highest wood flour dissolving capacity of those tested (Table I). It was also not surprising that the relatively high solubility of wood flour in these solvents coincided with a relatively high extracted solubilized lignin content, albeit in the presence of a large amount of the wood flour as a whole. Thus, there is relatively poor separation of the lignin from the cellulose. An interesting compromise between high lignin solubility and low wood flour solubility was achieved with [Emim][CH_3COO]. This solvent also provided very good extractability of the lignin; similar to $[Cl]^-$-containing ILs without the associated wood flour solubilization. This resulted in a relatively high extracted lignin content (4.4 g lignin/kg IL). It should be noted that the very low solubility of wood flour in [Emim][CH_3COO] contrasts with the high solubility of free cellulose. Specifically, while the former dissolves at <5 g/kg IL, we determined the solubility of microcrystalline cellulose to be >100 g/kg, which is similar to that reported by Zhao et al. (2008). Hence, the presence of lignin restricts the solubility of lignocellulose.

In addition to lignin and wood solubility and lignin extractability, we evaluated the enzymatic hydrolysis of native wood flour and wood flour pretreated with [Emim][CH_3COO] (Fig. 1). The native wood flour was rapidly hydrolyzed by *T. viride* cellulase, but this hydrolysis terminated after ca. 5 h at roughly 50% conversion of the available cellulose. Addition of fresh cellulase did not result in increased hydrolysis yield, indicating that the termination of the reaction was not due to loss of cellulase activity. Following pretreatment of 0.5 g wood flour in [Emim][CH_3COO] at 80°C for 24 h, over 90% of the available cellulose was hydrolyzed by *T. viride* cellulase in 5 h. ILs with poorer capacity to extract lignin, [Bzmim][Cl] and [Mmim][$MeSO_4$], supported a slightly higher reducing sugar content was generated by hydrolysis of the wood flours than for untreated wood flour; however, the yield of cellulose hydrolyzed was far lower than that for wood flour pretreated with [Emim][CH_3COO] (Fig. 1). These results are consistent with the incomplete accessibility of the native cellulose fraction of lignocellulose to cellulase, and thus limiting enzymatic hydrolysis. Removal of the lignin, via treatment with [Emim][CH_3COO], therefore, increases the accessible fraction of cellulose. Importantly, simply placing the wood flour in an IL that cannot extract sufficient amounts of lignin is insufficient to yield more biodegradable cellulose. As a result of these preliminary results, [Emim][CH_3COO] was selected as the IL to investigate

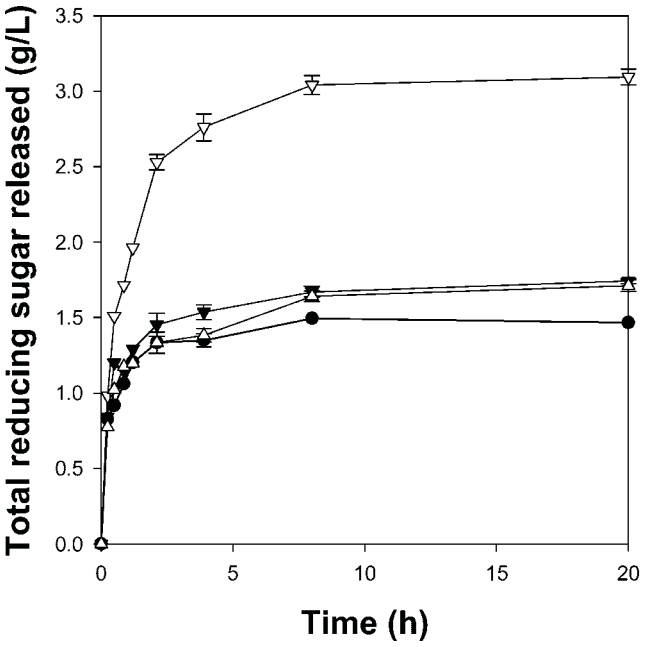

Figure 1. Effect of ionic liquids on the enzymatic hydrolysis of pretreated wood flour (●: untreated, ▽: [Emim][CH_3COO], ▼: [Bzmim][Cl], △: [Mmim][$MeSO_4$]). Reaction conditions: 10 mg pretreated wood flour, 3.5 mL of 50 mM citrate buffer (pH 4.7), 34 U/mL *Trichoderma viride* cellulase, 37°C, 200 rpm.

the selective extraction of lignin from lignocellulosic materials.

Influence of Pretreatment Temperature

The incubation temperature for pretreatment of wood flour was varied from 50 to 130°C (Table II). During pretreatment in [Emim][CH_3COO], the IL suspension turned dark brown presumably as a result of lignin extraction. The wood flour appeared to swell; however, it did not undergo substantial dissolution even after 90 min at 130°C. Following incubation for 90 min at various temperatures, the residual solid wood flour was removed and washed with water. A higher content of lignin was extracted and lower content of wood flour was recovered with increasing pretreatment temperatures (Table II), with approximately 63% of the initial lignin extracted after 90 min at 130°C. The total mass of extracted lignin and residual wood flour remained constant (ca. 17.5 mg) following pretreatment at all temperatures. The inability to close the initial mass balance (20 mg wood flour) is likely due to the loss of water from the wood flour into the IL and weighing errors that may be exacerbated following pretreatment with ILs. Similar results were obtained when 500 mg of wood flour was used in 10 g [Emim][CH_3COO], suggesting that the sample sizes employed were sufficient for the analyses performed. Pretreatment for 90 min at 130°C removed 16% and 26% of the cellulose and hemicellulose, respectively, the latter based on the wood flour xylan content. These results demonstrate that the wood flour underwent delignification with substantially smaller losses of cellulose and xylan upon pretreatment in [Emim][CH_3COO].

Figure 2a shows the time course of glucose released via the cellulase-catalyzed hydrolysis of wood flour pretreated for 90 min at temperatures ranging from 50 to 130°C. Both initial rate and extent of conversion after 11 h were enhanced by increasing the pretreatment temperature. Specifically, the initial rate of cellulose hydrolysis from wood flour by *T. viride* cellulase was 2.6 and 1.6 g/L h for pretreated (90 min at 130°C) and native wood flour, respectively. This increased cellulase reactivity translated into an increased enzyme-catalyzed conversion of cellulose hydrolyzed into glucose. While <50% of the native wood flour cellulose was

Table II. Effect of pretreatment temperature on composition and enzymatic hydrolysis of wood flour.

IL pretreatment[a]				Composition of IL-pretreated wood flour[b]				Enzymatic hydrolysis of IL-pretreated wood flour[c]	
Temperature (°C)	Extracted lignin (mg)[d]	Recovered wood flour (mg)[e]	Cellulose CrI	Cellulose (mg)	Xylan (mg)	Lignin (mg)[f]	Residues (mg)[g]	Released glucose (mg)	Digestibility[h] (%)
Untreated	0.0 (0%)	20.0 (100%)	63	10.3 (52%)	5.7 (28%)	3.5 (17%)	0.6 (2.8%)	5.2	46
50	0.8 (19%)	16.8 (84%)	57	8.7 (52%)	5.1 (31%)	2.8 (17%)	0.2 (0.9%)	5.8	50
70	0.9 (21%)	16.9 (85%)	52	9.0 (53%)	5.0 (30%)	2.7 (16%)	0.1 (0.7%)	6.7	58
90	1.2 (28%)	16.5 (83%)	47	9.0 (55%)	5.0 (30%)	2.5 (15%)	0.1 (0.3%)	9.2	80
110	1.9 (44%)	15.9 (80%)	38	8.7 (55%)	4.8 (30%)	1.9 (12%)	0.5 (3.2%)	10.4	90
130	2.7 (63%)	14.6 (73%)	30	8.7 (60%)	4.2 (29%)	1.3 (8.8%)	0.4 (2.7%)	10.9	95

[a]Two hundred and fifty milligrams of wood flour was incubated in 5 g [Emim][CH_3COO] for 90 min. Recalculated on the basis of 20 mg total wood flour.
[b]Recalculated on the basis of 20 mg total wood flour. Values in parentheses indicate composition percentages.
[c]Reaction conditions: recovered wood flour (mg)[e], 3.5 mL of 50 mM citrate buffer (pH 4.7), 34 U/mL *Trichoderma viride* cellulase, 37°C, 200 rpm, 11 h.
[d]Lignin content was determined with the Indulin AT standard. Values in parentheses represent extraction efficiency.
[e]Amounts used for enzymatic reactions. Values in parentheses represent percent of wood flour recovered relative to 20 mg untreated wood flour.
[f]Determined by NREL protocols 003 and 004 (NREL, 1996). Difference of lignin mass balance was caused by the difference between NREL method on residual wood flour solids and absorbance at 280 nm for kraft lignin standard.
[g]Protein, ash, and other sugars.
[h]Calculated on the basis of initial cellulose of untreated wood flour (10.3 mg).

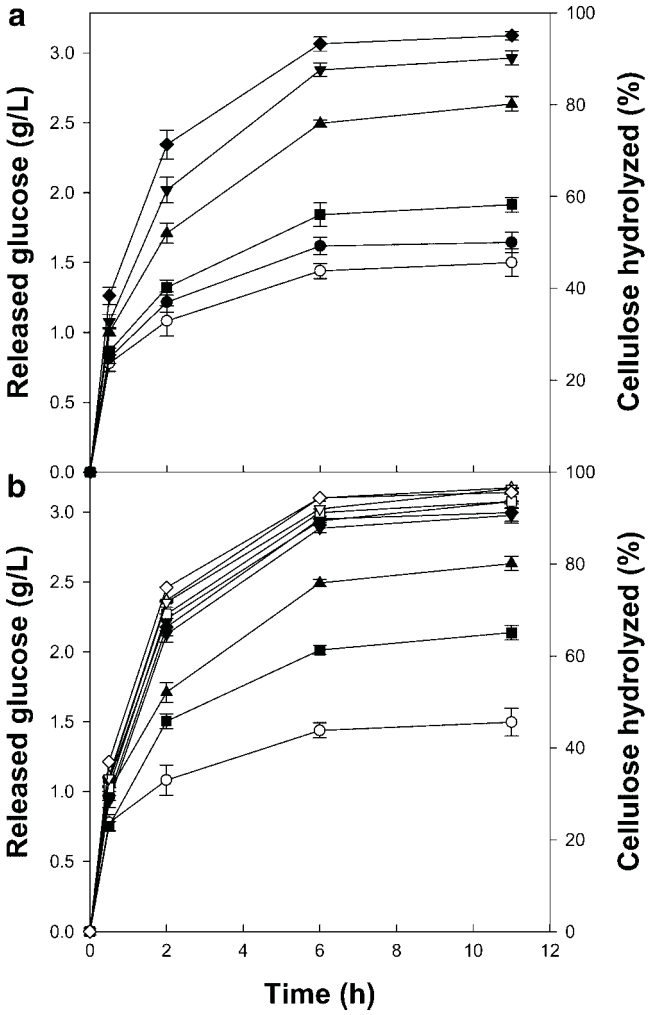

Figure 2. Influence of pretreatment variables on enzymatic hydrolysis of wood flour. **a**: Effect of pretreatment temperature (○: untreated, ●: 50°C, ■: 70°C, ▲: 90°C, ▼: 110°C, ◆: 130°C). **b**: Effect of pretreatment time (○: untreated, ■: 0.5 h, ▲: 1.5 h, ▼: 5 h, ◆: 8 h, ●:14 h, □: 19 h, △: 32 h, ▽: 42 h, ◇:70 h). Reaction conditions: recovered wood flour on the basis of initial 20 mg, 3.5 mL of 50 mM citrate buffer (pH 4.7), 34 U/mL *Trichoderma viride* cellulase, 37°C, 200 rpm.

hydrolyzed after 11 h, >95% was hydrolyzed following 90 min pretreatment at 130°C. Addition of fresh cellulase at 11 h to the native and various pretreated wood flour samples did not result in additional cellulose hydrolysis. The removal of lignin and the enhanced cellulolytic susceptibility of wood flour also coincided with a decrease in the CrI of the cellulose in the wood flour. Figure 3a depicts changes in the X-ray diffraction spectra of wood flour as a function of pretreatment temperature. Microcrystalline cellulose displayed an expected peak at $2\theta = 22.5^\circ$ corresponding to the (0 0 2) crystalline plane (Dadi et al., 2007). Untreated maple wood flour displayed a broader peak at $2\theta = 22.2^\circ$. After pretreatment for 90 min at increasing temperatures, the peak at 22.2° has both decreased in intensity and shifted to the left. Moreover, the peak between 14° and 16° essentially disappeared as pretreatment time increased. These diffraction pattern changes are consistent with a global decrease in the crystallinity of wood flour as a function of increasing pretreatment temperatures in [Emim][CH_3COO]. This loss in crystallinity was confirmed by calculating the CrI values (Table II), which revealed a clear decrease in CrI as a function of pretreatment temperature. These results demonstrate that [Emim][CH_3COO] can not only extract lignin but can also efficiently disrupt the crystal structure of cellulose in wood flour.

Influence of Pretreatment Time

To further understand the correlation of wood flour cellulose hydrolysis with lignin content and cellulose crystallinity, various samples of [Emim][CH_3COO]-pretreated wood flour were prepared by changing the incubation time in the IL. The incubation time for pretreatment of wood flour in [Emim][CH_3COO] was thus varied from 0.5 to 70 h at 90°C (Table III). No substantial solubility of wood flour was observed even after 70 h pretreatment. Increased pretreatment time progressively led to increased lignin extraction; ca. 40% of lignin was extracted from wood flour within 5 h and >85% was extracted after 70 h. Relatively little cellulose and hemicellulose was extracted up to 70 h pretreatment. Cellulose crystallinity decreased upon increased pretreatment times (Table III and Fig. 3b). After 70 h the cellulose ratio in pretreated wood flour slightly increased and the ratio of lignin dramatically decreased after pretreatment with [Emim][CH_3COO]. Figure 2b shows the time course of glucose released during cellulase-catalyzed hydrolysis of pretreated wood flour. Both initial rate (first 30 min) and conversion after 11 h were significantly increased with increasing pretreatment time up to ~32 h, yielding >90% cellulose hydrolysis.

Mechanism of Enhanced Cellulose Hydrolysis due to Wood Flour Pretreatment

Lignin is a major obstacle to enzyme attack on cellulose because it prevents enzyme accessibility (Chandra et al., 2007; Zhu et al., 2008) and can irreversibly adsorb cellulase (Ooshima et al., 1986). Indeed, our results confirm that a strong correlation exists between the fraction of cellulose hydrolyzed by cellulase and the fraction of lignin extracted from wood flour (Fig. 4a). Moreover, as shown in Figure 4a, >90% cellulose hydrolysis was affected by cellulase treatment even when the lignin content in the residual wood flour was 60% of that of the native lignocellulosic. This is a promising result as it suggests that it is not necessary to remove the majority of the lignin to achieve greater than 90% cellulose degradability. This also offers the possibility to process larger amounts of lignocellulosic materials.

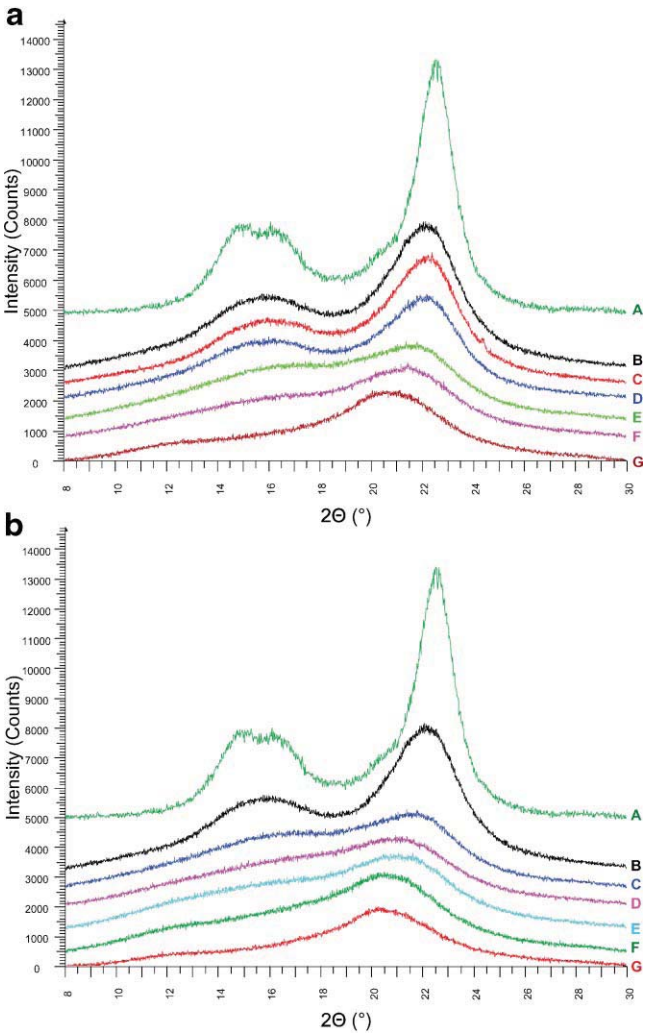

Figure 3. **a**: X-ray diffraction spectra of microcrystalline cellulose (A), untreated (B), and pretreated wood flour for 1.5 h at 50°C (C), 70°C (D), 90°C (E), 110°C (F), and 130°C (G). **b**: X-ray diffraction spectra of microcrystalline cellulose (A), untreated (B), and pretreated wood flour at 90°C for 1.5 h (C), 5 h (D), 19 h (E), 42 h (F), and 70 h (G). [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

It has long been reported that highly crystalline cellulose is less accessible to cellulase than amorphous cellulose. However, there is a lack of consistency in the relationship between cellulose crystallinity and enzymatic hydrolysis (Chang and Holtzapple, 2000; Zhu et al., 2008). However, our results show a strong correlation between cellulose crystallinity and effectiveness of enzymatic hydrolysis (Fig. 4b). This roughly paralleled the fraction of lignin extracted. Indeed, when 40% of the lignin was extracted, the cellulose CrI dropped below 45, resulting in >90% hydrolysis of the wood flour cellulose. There was a close correlation between lignin extraction and residual cellulose crystallinity (Fig. 4c).

Reuse of [Emim][CH_3COO] to Pretreat Wood Flour

A major disadvantage in using ILs as pretreatment solvents for lignocellulosic materials is their relatively high cost. Although many processes have been developed to decrease the production cost of ILs (Estager et al., 2007; Waterkamp et al., 2007), typical ILs remain expensive. Therefore, the reuse of ILs is important for commercial processing of biomass. In this work, [Emim][CH_3COO] was first used to pretreat wood flour and then washed with water to remove the extract from the residual wood flour solids. The water was removed by evaporation from the mixture of water and [Emim][CH_3COO], which contained mostly lignin. Without further purification, the [Emim][CH_3COO] solution was then reused to pretreat wood flour resulting in the accumulation of additional lignin. Although lignin continuously accumulated by repeating this procedure, the extraction efficiencies remained largely unaffected (Table IV). Subsequent extraction of lignin and reuse of [Emim][CH_3COO] are facilitated by the high solubility of lignin in [Emim][CH_3COO]. Cellulose hydrolysis of wood flour pretreated with [Emim][CH_3COO] that was reused five times was remarkably similar to wood flour pretreated

Table III. Effect of pretreatment time on composition and enzymatic hydrolysis of wood flour.

IL pretreatment[a]				Composition of IL-pretreated wood flour[b]				Enzymatic hydrolysis of IL-pretreated wood flour[c]	
Time (h).	Extracted lignin (mg)[d]	Recovered wood flour (mg)[e]	Cellulose CrI	Cellulose (mg)	Xylan (mg)	Lignin (mg)[f]	Residues (mg)[g]	Released glucose (mg)	Digestibility[h] (%)
Untreated	0.0 (0%)	20.0 (100%)	63	10.3 (52%)	5.7 (28%)	3.5 (17%)	0.6 (2.8%)	5.2	46
0.5	0.7 (16%)	17.2 (86%)	56	9.1 (53%)	5.1 (30%)	2.9 (17%)	0.1 (0.4%)	7.5	65
1.5	1.2 (28%)	16.5 (83%)	47	9.0 (55%)	5.0 (30%)	2.5 (15%)	0.1 (0.3%)	9.2	80
5.0	1.8 (42%)	16.0 (80%)	43	8.8 (55%)	4.9 (30%)	2.0 (13%)	0.3 (2.1%)	10.4	91
8.0	1.9 (44%)	16.1 (81%)	40	8.8 (55%)	5.0 (31%)	1.9 (12%)	0.3 (2.1%)	10.5	91
14	2.2 (51%)	16.0 (80%)	42	8.8 (55%)	5.0 (32%)	1.7 (11%)	0.5 (3.0%)	10.8	94
19	2.3 (54%)	15.9 (80%)	46	8.9 (56%)	5.0 (32%)	1.6 (10%)	0.4 (2.2%)	10.8	94
32	3.0 (70%)	15.6 (78%)	41	9.2 (59%)	5.1 (33%)	1.0 (6.6%)	0.2 (1.5%)	11.1	97
42	3.3 (77%)	15.2 (76%)	43	8.9 (59%)	4.7 (31%)	0.8 (5.2%)	0.8 (5.4%)	11.1	97
70	3.7 (86%)	14.7 (74%)	38	8.6 (59%)	4.4 (30%)	0.5 (3.2%)	1.2 (8.3%)	11.0	96

[a]Five hundred milligrams of wood flour was incubated in 10 g [Emim][CH_3COO] at 90°C. All other footnotes as described in the legend to Table II.

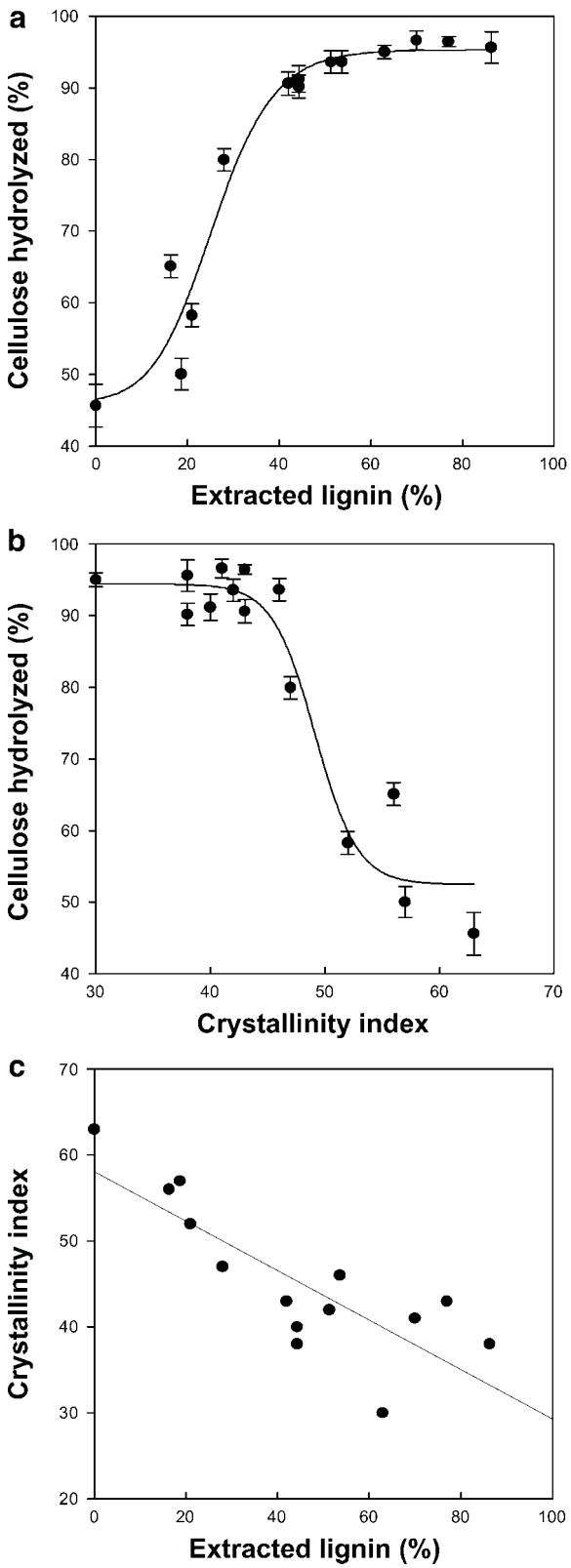

Figure 4. **a**: Relationship between lignin content extracted from wood flour and digestibility of pretreated wood flour. **b**: Relationship between the cellulose crystallinity of pretreated wood flour and digestibility. **c**: Correlation of lignin extraction and decreased cellulose crystallinity.

Table IV. Reuse of [Emim][CH_3COO] to pretreat wood flour.

Reuse of [Emim][CH_3COO][a]	Extracted lignin content (g/kg)[b]	Digestibility of pretreated wood flour (%)[c]
0	6.9	95.7
1st	14.2 (7.3)	92.1
2nd	21.3 (7.1)	92.7
3rd	28.4 (7.1)	92.7
4th	35.6 (7.2)	90.2

[a]Five hundred milligrams of wood flour was incubated in 10 g [Emim][CH_3COO] at 90°C for 24 h. Pretreated wood flour was washed with excess water and filtered. The filtrate was evaporated at 60°C for 6 h to remove water and then reused to pretreat wood flour.

[b]Determined by Indulin AT (kraft lignin) standard. Values in parentheses indicate incrementally extracted lignin for each reuse.

[c]Reaction conditions: pretreated wood flour 10 mg, 3.5 mL of 50 mM citrate buffer (pH 4.7), 34 U/mL *Trichoderma viride* cellulase, 37°C, 200 rpm, 24 h.

with pristine [Emim][CH_3COO]. The resulting solution of highly concentrated unmodified lignin thus obtained should be of value in preparing lignin-based products.

In conclusion, [Emim][CH_3COO] was used to decrease both lignin content and cellulose crystallinity of maple wood flour. [Emim][CH_3COO] did not dissolve the wood flour; however, facile extraction of the lignin was achieved. A strong inverse relationship was found between the effectiveness of cellulase-catalyzed hydrolysis of wood flour cellulose and both lignin content and cellulose crystallinity. Complete lignin removal was not needed and maximal cellulose degradability (>90%) was achieved even at 40% total lignin extracted. Finally, [Emim][CH_3COO] was reusable without significant loss in the yield of lignin extracted and subsequent cellulose hydrolyzed after four cycles. Furthermore, chemically unmodified lignin with high hydrophobicity could be easily precipitated from concentrated lignin solution in [Emim][CH_3COO] by adding excess water. This likely pristine maple wood lignin may prove useful as a raw material in the conversion to functional polymeric materials or as a starting point for catalytic transformation into liquid hydrocarbons. The tremendous chemical and physical breadth of ILs can be exploited in the design of new ILs with improved lignin extraction capability while providing a ready source of highly degradable cellulose and hemicellulose. Further studies, such as precipitation or extraction of lignin and removal or reuse of ILs by ion exchange, are needed to evaluate the efficient removal of the pristine lignin from the IL, thereby providing a new source for highly functionalized natural product.

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