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IONIC LIQUIDS ASSISTED ALKALINE FRACTIONATION ENHANCED TRIPLOID POPLAR BIOCONVERSION FOR BIOETHANOL PRODUCTION

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ABSTRACT

In this case, [Amim]Cl, [Bmim]Cl and [Emim]Ac were used to dissolve ball-milled triploid poplar, and the cellulose-rich preparations were subsequently recovered via incubation in 5 % NaOH aqueous solution and filtration. After the stepwise pretreatments, the carbohydrate content in the cellulosic residues increased to 73.3, 73.6 and 79.0 %, respectively, from 66.1 % in that with sole alkali fractionation. In comparison, the treatment with [Emim]Ac incurred transformation of cellulose I to II, which was favorable to enhance the alkaline fractionation for lignin extraction and disruption of biomass intact structure. After reconstitution, the digestibility of the three cellulosic preparations was all improved, yielding 1.3-fold higher fermentable sugars than that without IL pretreatment (67.2 %). These results indicated that the stepwise pretreatment with ionic liquid and alkali was effective for disrupting the intact structure of plant cell wall, and improving the productivity of bioethanol from lignocellulose biomass.

KEYWORDS: Bioethanol production; bioconversion; lignocellulosic biomass; ionic liquid.

INTRODUCTION

The worldwide demand for energy leads to a resurgence in the development of alternative energy. Biofuels, derived from biomass, are suggested as direct substitutes for fossil fuels in transport (Antizar-Ladislao and Tirrion Gomez 2008). In recent, the biomass contributes to 14 % of the world's primary energy demand (Veringa 2009). Furthermore, the International Energy Agency data indicates that the electricity generation from solid biomass in the European Union had been growing at an average rate of 2.5 % per year over the last decade (IEA 2009). Triploid poplar (*P. tomentosa*, 2n=3x=57, section *Populus*, family *Salicaceae*, genus *Populus*), is an indigenous species of white poplar. It has been widely used for landscape cultivation, ecological protection and the production of lumber and pulp in China. Due to the favorable growth rate, it can be harvested in 5 years (Zhu et al. 1995, 1998). The abundant biomass of triploid poplar makes it a potential source for energy production.

However, the recalcitrance of biomass limits its wider application. To achieve sustainable energy production, it will be necessary to overcome the chemical and structural properties that have evolved in biomass to prevent its disassembly (Himmel et al. 2007). Hence, effective pretreatment process is crucially required for breaking apart the highly ordered cellulose structure, and the shadow of hemicelluloses and lignin. Nowadays, the pretreatment strategies in cellulose-to-ethanol processes span over a wide range of reaction conditions, involving different pH values, temperatures, types of catalysts and holding times (Pedersen and Meyer 2010). Effective pretreatment should be able to improve the cellulose digestibility and to provide additional revenues for byproducts. Ionic liquids (ILs) are considered as effective pretreatment agents due to their ability of interaction with cellulose hydroxyl oxygen atoms via non-bonding or π electrons and preventing the cross-linking among cellulose molecules (Anderso et al. 2002, Zhang et al. 2005). Generally, ILs include salts of organic cations and anions, and the enormously potential ion combinations enable them to be designed and tuned (Freemantle 1998). The most successful cations for cellulose dissolution are based on the methylimidazolium and methylpyridinium cores with allyl-, ethyl-, or butyl- side chains, and the most favorable anions are found to be chloride, acetate and formate (Pinkert et al. 2009). Apart of cellulose, ILs are also capable of dissolving lignin and hemicelluloses, therefore, a special anti-solvent is necessary for the fractionation of hemicelluloses and lignin.

In this study, commercially available chloride-based and acetate-based ILs (1-allyl-3-methylimidazolium chloride ([Amim]Cl), 1-butyl-3-methylimidazolium chloride ([Bmim]Cl) and (1-ethyl-3-methylimidazolium acetate ([Emim]Ac)) were employed to dissolve wood flour of *P. tomentosa* due to their powerful dissolubility (Lee et al. 2009). The cellulosic preparations were recovered by alkaline aqueous solution fractionation. Structural characteristics and digestibility of the cellulosic preparations were comparably studied by composition analysis, CP/MAS ^{13}C NMR, XRD and enzymatic hydrolysis.

MATERIAL AND METHODS

Raw materials

Chips of *P. tomentosa* (3 years old) were obtained from Shandong Province, China. The wood powder was dewaxed with ethanol/toluene (1:2, v/v) for 8 h and then ball-milled for 48 h. The main components of the dewaxed material were determined as 44.5 cellulose, 19.3 xylan, 24.1 lignin and 3.2 % ash, basing on the standard NREL procedure [13]. The standard derivations were all less than 5 %.

Cellulose dissolution and regeneration

10 % (w/w) wood flour solutions were prepared by combining 1 g of sample with 10 g [Amim]Cl, [Bmim]Cl and [Emim]Ac (Sigma-Aldrich, St. Louis, MO) in 100 mL flasks, respectively. The mixtures were incubated in an oil bath at 130°C for 2 h with agitating. At the end of the incubation, 50 mL 5 wt % sodium hydroxide solution was added as anti-solvent to each wood/IL mixture with vigorous stirring. The precipitations were incubated at 75°C for one additional hour for fractionation. The cellulosic preparations were separated by filtration with filter paper, washed with distill water, freezing dried and labeled as CA, CB, and CE, respectively. Sample without the dissolving and generating process with ionic liquid was extracted with the same alkaline solution and the residue was labeled as C_0. All pretreatments were performed in duplicate.

Enzymatic hydrolysis of cellulose

A suspension solution of 200 mg sample in 10 mL sodium acetate buffer (50 mM, pH 4.8) was incubated in a water bath at 50°C for 48 h under gentle stirring.

The enzyme loadings were excessive 20 FPU/g (cellulase) and 30 CbU/g (β-glucosidase) (Novozyme Investment Co. Ltd.) in relation to the dry weight of cellulosic substrate for all hydrolysis experiments. The hydrolyzates were sampled periodically and analyzed by HPAEC system on a CarboPac PA 100 analytical column as described elsewhere (Yang et al. 2011). All enzymatic hydrolysis experiments were performed in triplicate, and average values and corresponding deviations were given.

Analysis methods

The main components of all samples were determined based on the standard NREL procedure Sluiter et al. (2012), and the monomeric sugars in the acid hydrolyzate were determined by a high-performance anion exchange chromatography (HPAEC, Dionex, ICS 3000, U.S.) system equipped with a CarboPac PA 20 analytical column. The analytic process was conducted according to a previous literature (Peng et al. 2010). The number-average (M_n) and weight-average molecular weights (M_w) of residual carbohydrates were determined by gel permeation chromatography (GPC) after tricarbanilation (Wood et al. 1986). 15 mg sample, anhydrous pyridine (4 mL) and phenyl isocyanate (1.0 mL) were added sequentially into test tubes, and then placed in an oil bath at 70°C for 48 h with agitation. Methanol (1.0 mL) was added to quench any remaining phenyl isocyanate at the end of reaction. The contents of each tube were then added dropwise to 100 mL 70 % (v/v) methanol aqueous solution to precipitate the derivatized carbohydrates. The solids were collected by filtration and successively washed with 50 mL 70 % (v/v) methanol aqueous solution and 100 mL water. The derivatized carbohydrates were then dried overnight under vacuum at 40°C. Prior to GPC analysis, the derivatized samples were dissolved in tetrahydrofuran (THF, 2 mg/mL), filtered through a 0.22 μm filter and placed in a 2 mL auto-sampler vial. The molecular weight distribution of the tricarbanilate samples were then analyzed on a PLgel 10 μ Mixed-B column featuring Agilent HPLC 1200 system equipped with a diode-array detector (DAD) at 240 nm using THF as the mobile phase (0.5 mL/min). The injection volume was 10 μL. The calibration curve was conducted based on four narrow polystyrene standards with average molecular weights of 1 030 000, 435 500, 156 000 and 66 000 g/mol (Polyer Laboratories Ltd., U.K.). All reported values for molecular weight and degree of polymerization were the average of triplicate determination.

X-ray powder diffraction in reflection mode was carried out using an XRD-6000 instrument (Shimadzu, Japan) with Ni-filtered Cu Kα radiation (χ = 1.54 Å), which operated at 40 kV and 30 mA. The scattering angle (2θ) was from 5 to 35° at a scanning speed of $2^{\circ}.min^{-1}$.

The crystalline index (*CrI*) was determined from the ratio of the separated crystalline peak area to the total area of crystalline and amorphous peaks (Segal et al. 1959). Cross-polarization/magic angle spinning (CP/MAS) ^{13}C NMR spectra of samples were obtained at 100.6 MHz using a Bruker AV-III 400M spectrometer (Germany). Dry cellulosic preparations were packed in a 4 mm zirconia (ZrO_2) rotor, and the measurements were performed using a CP pulse program with a 1 ms match time and a 2 s delay between transients. Spinning rate was 5 kHz.

RESULTS AND DISCUSSION

Recovery and chemical composition

Nakamura et al. (2010) have found that over 80 % cellulose can be recovered from cellulose/[C_2mim]Cl system by precipitation in various anti-solvents; however, the recovery rate of xylan is much dependent on the solubility of the anti-solvent for xylan. In the current study, the recovery of cellulose was neither significantly affected by the IL pre-dissolution process nor the types of ILs, varying in a narrow range of 70.8-76.3 % (Tab. 1).

Tab. 1: Recovery of wood flour, glucan and xylan during the pretreatment process.

	Recovery (%)*		
	Wood flour	**Glucan**	**Xylan**
C0†	77.6	76.3	54.4
CA	54.6	70.8	23.2
CB	56.4	71.1	19.9
CE	50.3	73.4	13.5

* The wood recovery represents the weight ratio of recovered substrate and starting raw material; the glucan and xylan recovery was calculated based on the sugar content listed in Tab. 2 as follow:

$$\text{Recovery (\%)} = \frac{\text{finial content of glucan (or xylan) in each sample} \times \text{the weight of sample}}{\text{initial content of glucan (or xylan) in the raw material} \times \text{the weight of material}} \times 100\ \%.$$

† C0 represents the cellulosic preparation obtained by the extraction with 5 % NaOH solution without IL treatment; CA, CB and CE represent the cellulosic preparations obtained by the treatment with [Amim]Cl, [Bmim]Cl and [Emim]Ac, and the sequential fractionation with 5 % NaOH solution at 75°C for 1 h, respectively.

In contrast, these factors exhibited the distinct effect on the xylan recovery, which was much lower than that of cellulose. These phenomena were probably attributed to the fact that the alkaline solution fractionated the xylan polymers from the wood-ILs mixture; however, the dissolved cellulose may self-assemble to a regularly lamellar structure as the IL molecules detached from cellulose during the regeneration (Li et al. 2009, Samayam 2011) and resist to the alkaline fractionation. In addition, the efficiency of regeneration has been found to be depended on the molecular weight. The dissolved high molecular weight components precipitated first in the anti-solvent (Leskinen et al. 2011). The much higher molecular weight of cellulose than xylan contributed to the considerable recovery of cellulose. In addition, xylan embeds between cellulose elementary fibrils and conglutinates them together (Jeoh et al. 2007). The ILs dissolution process disassembled the reticular structure of cell wall matrix, exposing more xylan to alkaline fractionation and introducing lower xylan recovery for C_A, C_B and C_E than C_0.

Tab. 2 depicts the chemical composition of the cellulosic preparations. Although 77.6 % wood flour was recovered after alkaline fractionation without IL pre-dissolution, glucan just accounted for 48.7 % in this preparation.

Tab. 2: Chemical composition of the cellulosic preparations (wt %).

	Sugar composition*						Lignin†	
	Rhamnose	**Arabinose**	**Galactose**	**Glucose**	**Xylose**	**Mannose**	**AI L**	**AS L**
$C_{0‡}$	0.1	0.5	0.4	48.7	15.7	0.7	21.5	4.3
C_A	0.2	0.4	0.5	62.1	9.5	0.6	20.0	1.5
C_B	0.1	0.4	0.3	64.5	7.9	0.4	18.9	3.1
C_E	ND§	0.3	0.2	72.2	6.0	0.3	12.4	1.5

* Standard deviation < 0.5%. † Standard deviation < 1%, AIL and ASL represent acid insoluble lignin and acid soluble lignin, respectively. ‡ Corresponding to the cellulosic preparations in Tab. 1,§ Not detectable.

The cellulose content was lower than that in a previous study, in which 85 % cellulose content was obtained from DMSO/water precipitating [Amim]Cl-pine wood chip solution (Wang et al. 2011). This result could be probably contributed to the speculation that the peeling reaction of cellulose in alkaline condition partially reduced the cellulose recovery. More cellulose was detected in the preparation of CE (72.2 %) than that in CA and CB (62.1 % and 64.5 %, respectively). This result was probably ascribed to the octagon-ring intermediate structure of cellulose-[Emim] Ac-water suggested by Liu et al. (2011), which reacted as a stereo-specific blockade for the interaction of hydroxyl ions with cellulose and then inhibited the peeling reaction. Moreover, the intermediate structure provided large vacuum between cellulose fibrils for alkaline extraction of xylan, incurring lower residual xylan in C_E (6.0 %) than that in C_A and C_B (9.5 and 7.9 %, respectively (Tab. 2). This phenomenon suggested that the anion could be tailored to having lone pair electrons, which can form hydrogen bonds with electron acceptors in a considerable size for improving cellulose recovery and xylan extraction with IL assisted alkaline fractionation.

XRD analysis

Fig. 1 depicts the X-ray profiles and crystalline indices of the cellulosic preparations. No distinct change between the crystalline indices of the ILs pretreated and the un-pretreated samples was observed.

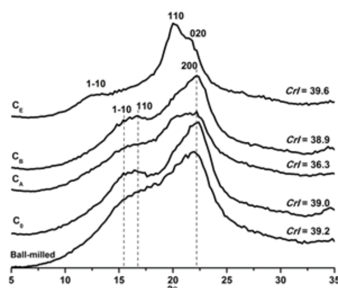

Fig. 1: XRD spectra of the ball-milled triploid poplar wood and cellulosic preparations.

The pronounced difference in the XRD diffractions was the typical cellulose II diffraction peaks in CE, indicating the transformation of cellulose configuration from cellulose I to II in CE. This phenomenon could be probably due to the mediation of different IL molecules. There was an intermediate structure in the pathway of re-crystallization to cellulose I, in which IL molecules presented and interacted with the hydroxyl groups displayed at the edges of the stacks; however, the IL molecules expulsed by anti-solvent and disordered cellulose re-crystallizes to cellulose II

directly without any intermediate structures (Samayman et al. 2011). The similarities observed in the spectra of the cellulosic preparations recovered from chloride-based ionic liquids suggested that the changing of cations introduced a minimal difference in the crystal of regenerated cellulose (Remsing et al. 2006). Comparing with chloride-based ILs, the [EMIM]Ac formed a ring between water, acetate and cellulose during the reconstitution, which prevented the formation of intermediate structure and prompted the re-crystallization to cellulose II. Alternatively, residual lignin is another factor impacts the re-crystallization of cellulose. Samayam et al. (2011) have reported that cellulose in high Tg lignin biomass retain a significant amount of cellulose I in IL pretreatment. It is assumed that the rigid lignin structure may initially orient the cellulose chains to re-assemble. The opposite polarities required for cellulose migration and conversion cellulose I to cellulose II is provided by lignin (Blackwell et al. 1978, Kerr and Goring 1975). Thus, the absence of a certain amount of lignin in CE also prompted the conformation of cellulose II. These phenomena demonstrated that [Emim]Ac was a favorable IL in disruption the intricate structure of biomass.

Molecular weight

ILs are believed to be capable of disrupting hydrogen bonds between different polysaccharide chains, making the carbohydrates more susceptible to chemicals and enzymes (Tadesse and Luque 2011). The effect of subsequence alkaline fractionation could be facilitated by the prior ILs dissolution. The dissociated cellulose chains provide active chain ends for the alkaline degradation. The effect of stepwise process with ILs and alkali on molecular weights of residual polysaccharides was elucidated. Sole alkaline fraction removed partial non-crystalline compounds with low molecular weights, resulting in a slight shift of peak to the higher molecular weight region (Fig.2) as compared to that of starting carbohydrates (ball-milled poplar flour).

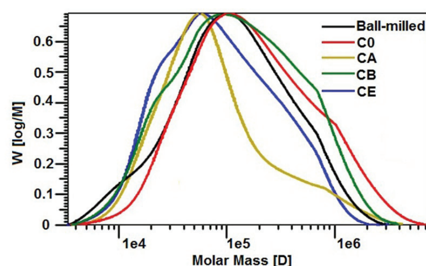

Fig. 2: Molecular weight distributions of the ball-milled triploid poplar wood and cellulosic preparations.

Low molecular weights were observed in the samples obtained from all ILs-assisted fractionation process, and C_B showed a slight higher molecular weight than C_A and C_E (Tab. 3), which was in agreement with the structural characterizations of these three fractions. This result might contribute to the more ordered portion in C_B than C_A and C_E indicated by XRD analysis. ILs pre-dissolution disrupted the crystalline structure of cellulose, incurring an increasing structural heterogen (as suggested by the polydispersities in Tab. 3).

Tab. 3: Molecular weights and polydispersities of the ball-milled triploid poplar and cellulosic preparations.

	Ball-milled sample	Cellulosic preparations*			
		C_0	C_A	C_B	C_E
Mw†	317000	381000	214000	258000	229000
Mn‡	60600	80700	42700	43700	53800
Mw/Mn§	5.2	4.7	5.1	5.9	4.3

* Corresponding to the cellulosic preparations in Tab. 1, † Represents weight-average molecular weight, ‡ Represents number-average molecular weights,§ Represents polydispersities.

However, the transformation of cellulose crystal in C_E did not significantly affect the molecular weight of this fraction comparing with the other two samples, but showed a narrower distribution. This result might be contributed to the lower residual lignin in C_E than that in C_A and C_B. Alternatively, the residual lignin consumed the derivation reagent, rendering un-complete derivation of carbohydrates and subsequently incurring the great polydispersity of sample.

Solid NMR spectroscopy

Solid-state NMR is a sufficient technology, which provides significant new insights regarding cellulose as well as lignin. Signals for cellulose locate in the region between 60 and 110 ppm, and peaks at about 152 and 56 ppm are corresponding to the aromatic ring and methoxyl groups in lignin, respectively (Fig. 3).

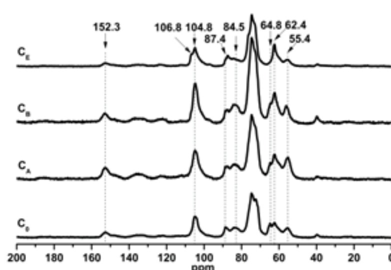

Fig. 3: Solid NMR spectra of the cellulosic preparation.

Further rationalization of the resonances for primary alcohol group and carbons anchoring the glycosidic linkage is crucially required to analyze the crystals of cellulose. Particularly, ordered and amorphous cellulose situate at different parts of the spectrum. The downfield wings of the C4 and C6 resonances are attributed to crystalline cellulose and the upfield signals of that are assigned to amorphous cellulose (Atalla and Vanderhart 1999). Peak for the amorphous template is more salient in the spectra of the regenerated preparations than that of C_0. The proposed dissolution mechanism of cellulose in ILs suggests that the separation of neighbored cellulose chains is introduced by the primary interaction between ILs and cellulose at the C-6 and C-3 hydroxyl groups (Remsing et al. 2006). This result interpreted that the interaction between ILs and hydroxyl groups effectively disrupted the hydrogen bonding interactions in the lignocelluloses. However, the different NMR spectrum of C_E indicated that the crystal of cellulose seemed to be significantly affected by the pretreatment with [Emim]Ac, coinciding with the results obtained from X-ray diffraction analysis. The disappearance of resonance from the crystalline cellulose in C6 and the splitting of C1 and C4 signals suggested that cellulose re-crystallized in a lattice similar to that of cellulose II after [Emim]Ac dissolution (Atalla

and Vanderhart 1999). Moreover, the low intensities at 152 and 56 ppm indicated that facile extraction of lignin was achieved with [Emim]Ac pretreatment. This result was in agreement with the chemical composition analysis, and also further confirmed that the pristine [Emim]Ac was more effective in improving the removal of lignin with alkali fractionation than [Amim]Cl and [Bmim]Cl.

Digestibility of cellulosic preparation

Regenerated cellulose is anticipated to allow a greater accessibility for the hydrolytic enzymes to rapidly penetrate and hydrolyze the cellulose [30]. It has also been hypothesized that regenerated cellulose has a large fraction of β-glucosidic bonds accessible to cellulase, due to the increased surface area and binding sites after the ILs pretreatment (Li et al. 2009, Samayam et al. 2011). The profiles of glucose liberation via enzymatic hydrolysis are shown in Fig. 4. A higher initial hydrolysis rate of regenerated samples than that of the un-regenerated one was observed, coinciding with the result reported by Dadi et al. (2009). This phenomenon was due to the fact that more accessible glycosidic bonds and chain ends made the formation of enzyme-substrate complex much easier for the reconstituted preparations than the un-restituted one (Zhao et al. 2009). Over 85 % reconstituted cellulose were converted to glucose at the end of the enzymatic hydrolysis, which was 1.3 times enhancements comparing with the cellulosic preparation from the raw material (67.2 %). Apart from the differences in crystallinity of cellulose, alterative possibility was that early conversion of regenerated cellulosic preparations to soluble oligomers allowed easy isolation and separation of the cellulase from the products for the second anchor (Gregg and Saddler 1996, Lu et al. 2002).

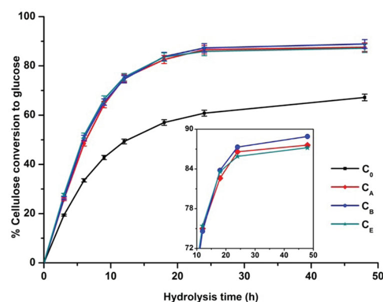

Fig. 4: Enzymatic hydrolysis of the cellulosic preparations. ((■) preparation without IL treatment; preparations by treatment with (♦) [Amim]Cl, (●) [Bmim]Cl and (*) [Emim]Ac, respectively).

Lignin is an obstacle to enzyme attack on cellulose and can irreversibly adsorb cellulose (Kumar and Wyman 2009). Although Cheng et al. (2011) have emphasized that the trend in hydrolysis rate for the biomass sample correlates with the conversion rates from cellulose I to cellulose II, the variability of lignin-carbohydrate complexes is another potential factor contributing to the recalcitrance of biomass. These interpretations could contribute to explain the similarity observed in the digestibility of cellulosic preparations with different crystals. In addition, this result also suggested that the alkaline fractionation process broke the lignin-carbohydrate complex and improved the cellulose digestibility, even though a certain amount of lignin still resided in the cellulosic substrate. Thus, it was not necessary to further remove the majority of lignin to achieve greater cellulose degradability.

CONCLUSIONS

IL assisted alkaline fractionation effectively disrupted the intact structure of plant cell wall and significantly increased the amenability of biomass to enzyme. Xylan with lower molecular weight than cellulose delayed precipitation and dissolved in alkaline solution, incurring a sharp decrease in xylan recovery with ILs dissolution. For the three kinds of ILs, higher carbohydrate component in C_E than that in C_A and C_B suggested that [Emim]Ac was more effective in promoting lignin removal than [Amim]Cl and [Bmim]Cl. Even though all the residues showed equal crystalline indices, a transformation of cellulose I to cellulose II during [Emim]Ac pretreatment was evidenced by XRD and NMR spectra.

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