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Characterization of ionic liquid pretreated aspen wood using semi-quantitative methods for ethanol production

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ABSTRACT

Aspen wood (*Populus tremula*) was pretreated with ionic liquid 1-ethyl-3-methylimidazolium acetate ([EMIM]OAc) and dilute sulfuric acid for improvement of ethanol production. The ionic liquid pretreatment included wood dissolution at 120 °C and 5% solid loading for 1, 3, and 5 h followed by regeneration using water as an anti-solvent. More than 95% enzymatic digestibility was achieved for the ionic liquid treated wood, while the yield from the untreated wood was only 5.3%. Furthermore, over 81% of the maximum theoretical ethanol yield was attained after 24 h fermentation of the ionic liquid treated wood, whereas the yields were only 5.3% and 42.1% for the untreated and dilute acid treated materials, respectively. A side-by-side comparative analysis of the pretreated materials using semi-quantitative techniques (e.g., Simons' staining and enzyme adsorption) revealed that the ionic liquid treatment was much more successful in increasing the cellulose accessibility to cellulases and decreasing the lignin content.

1. Introduction

Lignocellulose is the primary component in plant cell walls and is the most abundant sources of biomass in the world. It has a high potential to provide an inexpensive and renewable feedstock for the future production of bioethanol (Demirbas, 2011; Nigam & Singh, 2011; Sanchez & Cardona, 2008). Lignocelluloses have a complex and rigid structure, mainly composed of carbohydrate polymers (cellulose and hemicelluloses), lignin and other minor components. Cellulose is tightly bound to lignin and hemicellulose through hydrogen and covalent bonding in the matrix structure of the cell wall (Seeta Laxman & Lachke, 2009; Taherzadeh & Karimi, 2008a). Saccharification, the process of breaking down cellulose into simple sugars, is an essential stage to allow the conversion to bioethanol. However, the recalcitrant nature of lignocellulose limits its chemical and biological degradation, and thus hinders the hydrolysis process (Taherzadeh & Karimi, 2007, 2008b).

The major obstacle of hydrolysis of lignocellulose is not only due to the protective nature of lignin and hemicelluloses, but also the intrinsic physical and morphological characteristics of the heterogeneous biomass such as cellulose fibers crystallinity and surface area, which govern the hydrolysis efficacy. The cellulose crystallinity is defined as the ratio of the crystalline to the amorphous portions that influences the rate of hydrolysis, given that the loosely organized amorphous cellulose is more amiable to enzymatic (cellulase) conversion than the closely packed crystalline cellulose. The available surface area (exterior and interior), porosity, and range of pore sizes play significant roles in the rate and extent of enzymatic hydrolysis, by providing active sites for cellulase adsorption and facilitating enzyme diffusion into the pores (Alvira, Tomas-Pejo, Ballesteros, & Negro, 2010; Chandra, Ewanick, Hsieh, & Saddler, 2008; Chandra, Esteghlalian, & Saddler, 2008).

Pretreatment is a key step to overcome the lignocelluloses recalcitrance and make them more susceptible for hydrolysis by cellulases. Several methods have been discussed in the literatures including physical (e.g., mechanical comminution and pyrolysis), chemical (e.g., acid hydrolysis and organosolv process), physicochemical (e.g., steam explosion and AFEX), and biological pretreatments (e.g., fungi and actinomycetes). One of the primary challenges is the development of a cost-effective pretreatment technology, while remaining environmental and energy efficient. Some pretreatment techniques such as acid hydrolysis are hazardous, corrosive, and need neutralization due to the formation of inhibitory compounds, whereas others like steam explosion and AFEX are capital intensive (Chandra et al., 2007; Gupta, Khasa, & Kuhad, 2011; Taherzadeh & Karimi, 2008b; Yang & Wyman, 2008).

Certain ionic liquids have demonstrated a promising ability for efficient dissolution of biomass and its cellulose regeneration upon

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dilution with an anti-solvent (Diedericks, van Rensburg, Garcia-Aparicio, & Gorgens, 2012; Geng & Henderson, 2012; Li et al., 2010; Moniruzzaman & Ono, 2012; Tan & Lee, 2012; Uju et al., 2012). These ionic solvents are liquid at or near room temperature, and typically are composed of large organic cations and small inorganic anions. Some ionic liquids are green solvents, and have several attractive properties, such as excellent chemical and thermal stability, non-flammability, and very low to negligible vapor pressure (Zhu et al., 2006). Pretreatment with ionic liquids can be applied at moderate conditions with the possibility of almost complete recovery of the solvent for subsequent reuse (Zhao et al., 2009). Among the ionic liquids, 1-alkyl-3-methylimidazolium salts such as 1-ethyl-3-methylimidazolium acetate ([EMIM]OAc) and 1-butyl-3-methylimidazolium ([BMIM]Cl) are found to be very efficient solvents for cellulose dissolution (Auxenfans et al., 2012; Mora-Pale, Meli, Doherty, Linhardt, & Dordick, 2011). It has been reported that out of six different ionic liquids, [EMIM]OAc significantly enhanced sugarcane bagasse enzymatic saccharification yield over 90% (Sant'Ana da Silva, Lee, Endo, & Bon, 2011). Furthermore, [EMIM]OAc does not have a reactive side group, and is non-toxic, non-corrosive, and biodegradable (El Seoud, Koschella, Fidale, Dorn, & Heinze, 2007; Zhu et al., 2006). Despite the high potential of ionic liquids, there is limited knowledge dealing with effects of ionic liquids on features of lignocellulosic substrates that promote their hydrolytic susceptibility.

In order to track the changes in lignocellulose properties due to the pretreatment, several quantitative methods such as nitrogen adsorption and solute exclusion have been used to measure the substrate surface area and accessibility to cellulases (Chandra, Ewanick, et al., 2008; Chandra, Esteghlalian, et al., 2008). There are also a few reports on application of some more quick methods such as Simons' stain technique and water retention value. The Simons' stain method was originally developed as a staining method for evaluation of the mechanical damage of pulp fibers during the beating process. However, it has shown levels of success in estimating a given pretreated substrate's susceptibility to enzymatic hydrolysis, and thus the effectiveness of the pretreatment (Chandra, Ewanick, et al., 2008; Chandra, Esteghlalian, et al., 2008).

This research evaluated the effectiveness of ionic liquid [EMIM]OAc pretreatment of aspen wood compared to dilute sulfuric acid by assessing the changes in physiochemical and structural properties of lignocellulosic substrate. The research focused on the use of rapid semi-quantitative methods to gain a better understanding of the key factors affecting enzymatic digestibility and subsequent bioconversion to ethanol.

2. Materials and methods

2.1. Raw material

An aspen wood (*Populus tremula*) log was harvested on summer 2011 from a local forest (Mazandaran, Iran, 36°28′N, 51°54′E, 480 m). The sample was debarked, cut into small pieces, and dried at room temperature. The wood chips were milled and screened to achieve a particle size in the range of 177–840 μm. Moisture content of the wood was determined by drying samples in duplicates at 105 °C for 24 h.

2.2. Pretreatments

2.2.1. Ionic liquid pretreatment

Ionic liquid, 1-ethyl-3-methylimidazolium acetate ([EMIM]OAc), produced commercially by BASF (Ludwigshafen, Germany), was purchased from Sigma–Aldrich (St. Louis, MO, USA). An amount of 1 g of the aspen wood (dry basis) was thoroughly mixed with 19 g of the ionic liquid in 50 ml pressure glass bottles at 120 °C for increasing time periods (1, 3, and 5 h). After dissolution of the aspen wood in the ionic liquid for different pretreatment times, an equal volume of deionized water was added into the wood solutions to stop the treatment and precipitate the cellulose under vigorously stirring condition. The regenerated materials were filtered (Whatman Grade No. 41) under vacuum pressure and washed with boiling water until a clear filtrate was appeared. All pretreated materials were then oven dried at 40 °C for 48 h, weighed, and stored in sealed bags at 4 °C until use.

2.2.2. Dilute sulfuric acid pretreatment

The aspen wood was presoaked in dilute sulfuric acid solution (1.2%, w/w) at a solid loading of 5% (w/w) and room temperature for 4 h. The slurry was heated up (10 °C/min) to reach the final temperature of 120 °C and then isothermally kept at 120 °C for 60 min (Pinto & Kamden, 1996). After pretreatment, the mixture was filtered under vacuum pressure, and the recovered materials were washed by distilled water to reach a pH of 6.0. The pretreated materials were oven dried at 40 °C for 48 h, weighed, and kept in sealed bags at 4 °C until use.

2.3. Materials characterization

2.3.1. Composition analysis

Polymeric carbohydrates, acid soluble lignin, and acid insoluble lignin contents of the untreated and pretreated wood were determined by the method presented by the National Renewable Energy Laboratory (Sluiter et al., 2008).

2.3.2. Simons' stain method

Accessibility of the woody materials to the cellulases was assessed by evaluating the pore size distribution of the samples using Simons' stain method. This method is based on the competitive adsorption of two dyes, Direct Blue 1 (DB) and Direct Orange 15 (DO), in an aqueous medium, and in this study, a modified version of Simons' stain technique was used (Chandra, Ewanick, et al., 2008; Chandra, Esteghlalian, et al., 2008). DB (Pontamine Fast Sky Blue 6BX) and DO (Pontamine Fast Orange 6RN) dyes were provided by Pylam Products (Garden City, NY). The low-molecular-weight (LMW) fraction of the DO was filtered through a 100k molecular mass cut-off membrane, and the retentate was collected to make the DO stock solution. Approximately, 100 mg of the treated or untreated wood was weighed into 15 ml test tubes, and 1.0 ml of phosphate buffered saline solution (pH 6, 0.3 M PO_4, 1.4 mM NaCl) was added to each tube. Different amounts of DB and DO stock solutions (10 mg/ml), ranging from 0.25 to 2 ml, were added to the tubes and diluted up to 10 ml with distilled water, resulting in a ratio of 1:1 DO and DB at increasing concentrations. All the tubes were incubated at 70 °C and 150 rpm for 15 h and then centrifuged at 7000 × *g* for 5 min. The amounts of DO and DB adsorbed by the wood was calculated using the difference between the initial and final dye concentration in the supernatant solution measured by UV-Visible spectrophotometer at wavelengths of 624 and 455 nm, respectively (Chandra et al., 2009; Esteghlalian, Bilodeau, Mansfield, & Saddler, 2001). The spectrophotometer was calibrated by preparing standard curves of each dye and measuring the absorbance at 455 and 624 nm.

2.3.3. Water retention value

Water retention value (WRV) is the ability of a substrate to keep water molecules in the cell wall pores and is used to represent the total volume of the pores. The WRV measurement is based on subjecting a water-saturated sample to a centrifugal force, and the WRV is defined as the amount of retained water per unit weight of dry material. Approximately 1 g of the substrate was mixed with

Table 1
Chemical composition (%) of untreated and pretreated aspen wood.[a]

Component	Untreated aspen wood	Dilute acid pretreated aspen wood	[EMIM]OAc pretreated aspen wood (time)		
			1 h	3 h	5 h
Glucan	49.0	56.1	59.3	60.4	63.9
Xylan	14.9	7.5	9.9	9.2	8.6
Mannan	2.0	1.9	1.7	1.5	1.7
Galactan	0.5	ND[b]	ND	ND	ND
Arabinan	0.8	ND	ND	ND	ND
Acid soluble lignin	1.0	0.8	1.1	1.0	1.0
Acid insoluble lignin	24.6	25.3	20.8	19.2	16.4

[a] The data are average values of the two replications.
[b] Not detectable.

deionized water at 150 rpm for 60 min. The mixture was filtered using a nylon membrane (45 μm), and the filter cake was transferred into a small bag made of nonwoven materials and soaked in deionized water at room temperature for 2 h. The bag was wrapped, placed into a centrifuge tube with support to make space for water accumulation, and centrifuged at 3000 × *g* for 15 min. The substrate was collected and weighed before and after drying at 105 °C for 24 h. The WRV was calculated as follows (Luo & Zhu, 2011; Program, 2000):

$$\text{WRV}\ \ (\text{g/g dry material}) = \frac{W_{\text{wet}} - W_{\text{dry}}}{W_{\text{dry}}} \quad (1)$$

where W_{wet} and W_{dry} are respectively the wet and oven dry mass of the material.

2.3.4. Substrate accessibility to cellulase

The maximum extent of enzyme adsorption, an indication of the available surface area for cellulase binding, was measured according to the method presented by Kumar and Wyman (2009) with modifications as follows. Different amounts of enzyme were dissolved in 5 ml sodium citrate buffer (50 mM, pH 4.8 ± 0.1) and added to the pretreated and untreated substrates to make suspensions with a final dry solid content of 2% (w/v). The mixtures were continuously stirred and preserved at 4 °C to avoid changes in substrates properties due to the possible hydrolysis. After 90 min, the supernatants were collected and centrifuged at 5000 × *g* for 15 min. The free protein content of the supernatants was measured by Bio-Rad protein assay (Richmond, CA, USA) with bovine serum albumin as a protein standard. The bonded protein was indirectly calculated by subtracting the measured free protein from the total protein added. The Langmuir isotherm model was used to describe the equilibrium adsorption behavior according to the following equation:

$$E_a = \frac{E_m K_d E_f}{1 + K_d E_f} \quad (2)$$

where E_a is the concentration of bound protein (mg protein/g substrate), E_m is the maximum adsorption capacity based on complete monolayer coverage assumption (mg protein/g substrate), E_f is the free protein concentration in the supernatant (mg protein/ml), and K_d is the binding constant (ml/mg protein). The Langmuir parameters (E_m and K_d) were determined by non-linear regression of the experimental adsorption data based on the least-square method using Curve Fitting Toolbox of MATLAB software (Version R2011a; Math Works Inc., MA, USA).

2.3.5. FTIR spectroscopy

Fourier transform infrared (FTIR) spectroscopy technique was applied to examine changes in structure of the woody materials caused by the pretreatments. The FTIR system (Tensor 27 FT-IR Spectrometer, Bruker, Germany) was equipped with a universal attenuated total reflection sampling accessory, and deuterated triglycine sulfate detector. The spectra were acquired at 4 cm^{-1} resolution with an accumulation of 60 scans per sample, and recorded in the range of 600–4000 cm^{-1}.

2.3.6. SEM

Scanning electron microscopy (SEM) analysis was performed to obtain detailed microscopic view of morphologic changes occurred by the pretreatments. Prior to the study, the untreated and pretreated samples were coated with gold (BAL-TEC SCD 005), and then images were recorded with an acceleration voltage of 15 kV (Philips XL30).

2.3.7. Enzymatic digestibility of the substrate

Two commercial enzymes, cellulase from *Trichoderma reesei* (Celluclast 1.5 L, 90 FPU/ml, Sigma) and cellobiase from *Aspergillus niger* (Novozyme 188, 744 CBU/ml, Sigma), were used for hydrolysis experiments. Enzyme activities were determined according to standard IUPAC procedures (Ghose, 1987). Enzymatic digestibility of untreated and pretreated materials was conducted in 50 mM sodium citrate buffer (pH 4.8 ± 0.1) at 3% (w/v) solids concentration, 50 ± 1 °C, and 150 rpm for 72 h. Enzymes were loaded at 20 FPU cellulase and 30 CBU β-glucosidase per gram glucan and the suspension was supplemented with 0.5 g/l sodium azide to restrict any microbial growth during the hydrolysis (Goshadrou, Karimi, & Taherzadeh, 2011). Samples were taken periodically over 72 h, immersed in boiling water for 5 min to deactivate the enzymes, and stored at −20 °C before sugar analysis. Pure cellulose (Avicel PH-101, Sigma) as a control was run alongside the samples in the hydrolysis experiments. The amount of glucose released during digestibility experiments was determined enzymatically using glucose oxidase-peroxidase assay kit (Sigma). The substrate enzymatic digestibility was defined as:

$$\text{Enzymatic digestibility}\ \ (\%) = \frac{\text{Produced glucose}\ \ (\text{g/l}) \times 100}{\text{Substrate concentration}\ \ (\text{g/l}) \times F \times 1.111} \quad (3)$$

where F is the biomass glucan fraction (Table 1), and the constant 1.111 is used for conversion of glucan to glucose.

2.4. Microorganism and media

A flocculating strain of *Saccharomyces cerevisiae* CCUG 53310 (Culture Collection University of Göteborg, Göteborg, Sweden) was used for ethanol production. The yeast was cultivated on agar plates contained (g/l): glucose, 20; peptone, 20; and yeast extract 10. After two days of incubation at 30 ± 0.5 °C, the plates were stored at 4 °C until use.

2.5. Inoculums preparation

The biomass for fermentation was prepared in 250 ml cotton-plugged Erlenmeyer flasks containing 50 g/l glucose and

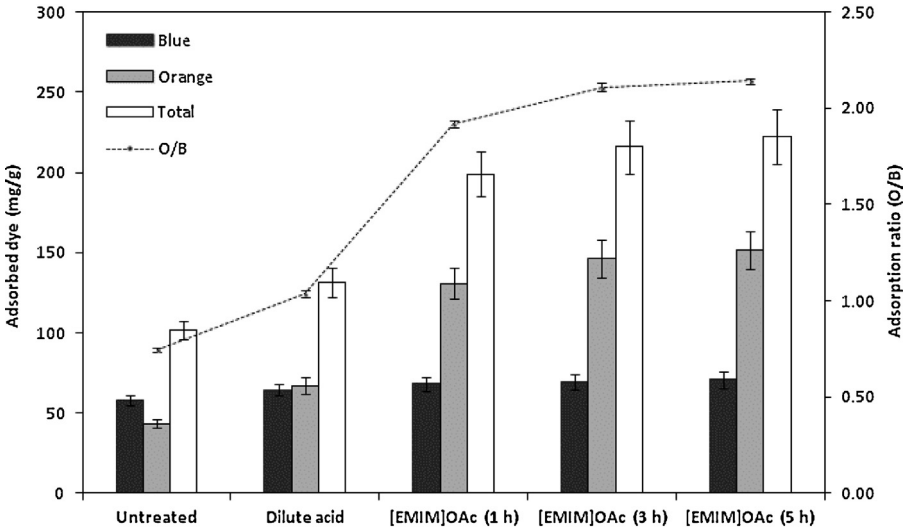

Fig. 1. Dyes adsorption during Simons' stain analysis for untreated and pretreated aspen wood.

necessary nutrients including (g/l): yeast extract, 5; $(NH_4)SO_4$, 7.5; K_2HPO_4, 3.5; $MgSO_4{\cdot}7H_2O$, 0.75; $CaCl_2{\cdot}2H_2O$, 1; in distilled water (Goshadrou, Karimi, & Taherzadeh, 2013). The pH was adjusted to 5.0 ± 0.1 using either 2.5 M sodium hydroxide or 0.5 M sulfuric acid. The media was autoclaved at 121 °C for 20 min, inoculated by adding a single colony of the yeast and then incubated for 30 h at 30 ± 0.5 °C and 150 rpm. The produced biomass was then aseptically harvested by centrifugation at $3400 \times g$ for 15 min (D-78532 Tuttlingen, Hettrich, Germany), and the supernatant was removed.

2.6. Bioconversion to ethanol

The sugar solutions following 30 h enzymatic hydrolysis of the untreated and pretreated aspen wood (without sodium azide addition) were supplemented with necessary nutrients (g/l): yeast extract, 5.0, $(NH_4)_2SO_4$, 7.5, $MgSO_4{\cdot}7H_2O$, 0.75, K_2HPO_4, 3.5, and $CaCl_2{\cdot}2H_2O$, 1.0. The media were autoclaved at 121 °C for 20 min, cooled to room temperature, and aseptically inoculated with 5 g/l yeast biomass (based on the dry mass). The samples were incubated at 35 ± 0.5 °C and 150 rpm for 30 h. All fermentations were performed in 115 ml serum bottles sealed with rubber stoppers. The bottles were purged with pure nitrogen at the beginning of the cultivation and vented with syringe needles to release the CO_2 during fermentation. Ethanol yield was calculated as a percentage of maximum theoretical yield according to the following equation:

$$\text{Ethanol yield (\%)} = \frac{\text{Produced ethanol (g/l)} \times 100}{\text{Substrate concentration (g/l)} \times F \times 1.111 \times 0.51} \quad (4)$$

The factor 0.51 was applied to consider the theoretical conversion of glucose to ethanol.

2.7. Analytical methods

The liquid samples from fermentation were analyzed using an HPLC system equipped with UV-Vis and refractive index detectors (Jasco International Co., Tokyo, Japan). The ethanol concentration was determined after separation on an Aminex HPX-87H ion-exchange column (Bio-Rad, Richmond, CA, USA) at 60 °C with 0.6 ml/min eluent of 5 mM H_2SO_4. The concentration of the sugars in composition analysis (Section 2.3.1) was analyzed using an ion-exchange Aminex HPX-87P column (Bio-Rad, Richmond, CA, USA) at 85 °C with 0.6 ml/min ultra-pure water as eluent.

All the analyses and experiments were performed in duplicate, and the reported data are average values of the two replications.

3. Results

3.1. Effect of pretreatment on aspen wood composition

The main chemical composition of control (untreated), dilute sulfuric acid, and ionic liquid pretreated aspen woods were analyzed, and the results are presented in Table 1. The pretreatments resulted in materials with different cellulose and hemicellulose contents. The untreated wood contained 49.0% glucan which increased to 56.1% and 59.3–63.9% after dilute acid and ionic liquid pretreatments, respectively. All the pretreatments removed the xylan and other polysaccharides and this was more pronounced after using the dilute acid pretreatment. The acid soluble lignin content of the wood was low (1.0 ± 0.1%) and remained unchanged after the ionic liquid pretreatments, while it decreased to 0.8% by the dilute acid pretreatment. The acid insoluble parts of lignin were reduced from 24.6% to 16.4–20.8% during ionic liquid treatments, while it increased slightly after dilute acid treatment. Increasing ionic liquid pretreatment time highly impacted the lignin removal yield.

3.2. Effect of pretreatment on surface area and pore sizes

The two-color Simons' stain experiments were carried out on the untreated and pretreated woody materials to quantify changes in the biomass porosity and pores sizes as a result of the pretreatments. The results (Fig. 1) indicated that the total adsorbed dyes increased from 101 to about 130 mg/g using dilute acid treatment, whereas the extent of adsorption was significantly higher for biomass treated with ionic liquid, ranging from 199 to 222 mg/g for 1 and 5 h treated materials, respectively. Adsorption ratio (DO/DB) is defined as the maximum adsorbed DO by the substrate over that of DB. The adsorption ratio was measured as 0.75 for untreated aspen wood, while it increased to 1.92, 2.11, and 2.14 after 1, 3, and 5 h pretreatment with the ionic liquid, respectively. No significant difference between 3 and 5 h pretreatment times was detected. Among the pretreated woods, the lowest ratio of 1.04 was observed for the biomass treated with dilute acid.

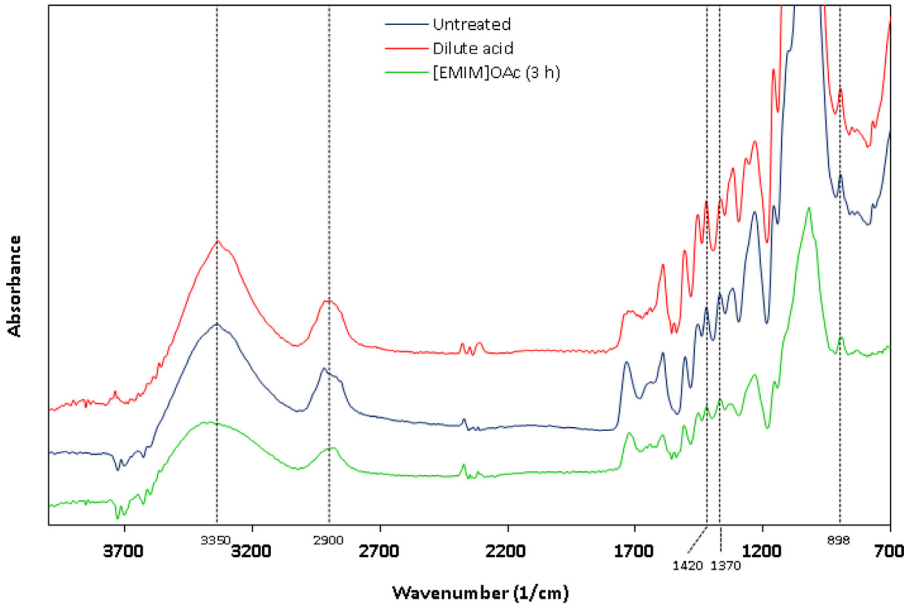

Fig. 2. FTIR spectra of untreated and pretreated aspen wood.

3.3. Effect of pretreatment on pore volumes

The swelling ability of the biomass before and after pretreatments was characterized by the water retention value as it mainly reflects the pores volume of a substrate. For determination of WRV values, the substrate was soaked in deionized water, subjected to centrifugal force, oven-dried, and then the ratio of absorbed water to dry fibers was calculated. As shown in Table 2, WRV of the untreated aspen wood was 0.71 g/g dry material, which significantly increased up to 1.87 g/g dry material after the ionic liquid treatment. The extent of water swelling capacity was less affected by ionic liquid treatment time. Unexpectedly, the acid pretreated wood exhibited the least ability to absorb water molecules even in comparison with untreated aspen wood.

3.4. Effect of pretreatment on accessible surface area

Langmuir adsorption isotherm was applied to describe cellulase binding to the untreated and pretreated substrates, and the maximum extent of adsorbed protein was considered proportional to the surface area available for the cellulase enzyme. Adsorption capacity (E_{max}), affinity parameter (K_d), and binding strength (S) are listed in Table 3, along with the determination coefficient (R^2) in the range of 0.958–0.967. As shown in Table 3, the adsorption capacity of the aspen wood was 101.6 mg/g, which was increased to 121.6–176.8 mg/g after the pretreatment. Among the pretreated materials, the solids remained after the dilute acid treatment showed the lowest adsorption capacity (121.6 mg/g solids), whereas 5 h ionic liquid treated wood was the most accessible substrate to the cellulase. However, no significant improvement in cellulase adsorption behavior was observed between samples treated with ionic liquid for 3 and 5 h. The binding affinity of cellulase to the samples increased from 1.01 to 1.12–1.38 ml/mg after pretreatment using dilute acid and ionic liquid, respectively.

Table 2
Water retention values (WRV) of untreated and pretreated aspen wood.

Substrate	WRV[a] (g/g dry material)
Dilute acid treated	0.67
[EMIM]OAc treated (1 h)	1.81
[EMIM]OAc treated (3 h)	1.85
[EMIM]OAc treated (5 h)	1.87
Untreated	0.71

[a] The data are average values of the two replications.

3.5. Effect of pretreatment on substrate crystallinity

The bands for untreated and representative treated materials were observed over the range of 600–4000 cm^{-1}. The absorbance spectra and characteristic of the bands are presented in Fig. 2 and Table 4, respectively. The band intensities associated with lignin at 1240 cm^{-1} (C—O vibration in syringyl and guaiacyl condensed lignin), 1460 cm^{-1} (asymmetric bending in $C—H_3$ in lignin), 1510 cm^{-1} (aromatic skeleton C=C stretching in lignin), and 1590/1627 cm^{-1} (C=C stretching vibration in lignin) significantly decreased for the 3 h ionic liquid treated materials compared to untreated aspen wood, whereas the dilute acid treated solid showed higher lignin content than the other woody materials.

Table 3
Langmuir isotherm parameters for cellulase binding to untreated and pretreated materials.

Substrate	Langmuir isotherm parameters		$S = E_{max} \times K_d$ (ml/g substrate)	R^2
	E_{max} (mg/g substrate)	K_d (ml/mg)		
Aspen wood				
Untreated	101.6	1.01	102.6	0.958
Dilute acid treated	121.6	1.12	136.2	0.967
[EMIM]OAc treated (1 h)	169.9	1.37	232.8	0.964
[EMIM]OAc treated (3 h)	175.4	1.38	242.0	0.961
[EMIM]OAc treated (5 h)	176.8	1.35	238.7	0.963
Pure cellulose	88.9	0.34	30.2	0.966

Table 4
Characteristic of bands in FTIR spectra of untreated and pretreated materials.

Wavenumber (cm^{-1})	Functional group	Assignment	Intensity		
			Untreated	Dilute acid	[EMIM]OAc (3 h)
1055	C—O stretching	Cellulose and hemicellulose	0.680	0.938	0.284
1158	C—O—C asymmetric stretching	Cellulose	0.300	0.414	0.143
1240	C—O stretching	Hemicellulose and lignin	0.294	0.340	0.151
1460	Asymmetric bending in $C—H_3$	Lignin	0.176	0.263	0.110
1510	C=C stretching of the aromatic ring	Lignin	0.140	0.226	0.098
1590	C=C stretching	Lignin	0.147	0.211	0.088
1627	C=C stretching	Lignin	0.115	0.165	0.076
1730	C=O stretching of acetyl or carboxylic acid	Hemicellulose and lignin	0.136	0.161	0.086
3350	O—H stretching	Cellulose	0.175	0.231	0.100

Aspen wood	LOI (A1420/A898)[a]	TCI (A1375/A2900)[a]
Dilute acid treated	1.88	0.98
[EMIM]OAc treated (3 h)	1.12	0.82
Untreated	1.66	1.05

[a] The crystallinity indices were determined following the method of Nelson and O'Connor (1964).

Furthermore, the spectral ratios A1420/A898 and A1370/A2900, which are respectively referred as crystallinity index or lateral order index (LOI) and total crystallinity index (TCI), were calculated (Carrillo, Colom, Sunol, & Saurina, 2004; Nelson & O'Connor, 1964; Uju et al., 2012). Both LOI and TCI indices were efficiently impacted by the ionic liquid pretreatment and dropped from 1.66 and 1.05 to 1.12 and 0.82, respectively. However, the total crystallinity index was less impacted by the dilute acid pretreatment and reduced to 0.98. Besides, LOI of the acid treated materials (1.88) showed that the pretreatment resulted in slightly higher crystallinity than the untreated biomass. The bands related to intra- and intermolecular O—H stretching of cellulose hydroxyl group can be detected in the range of 3000–3600 cm^{-1} (Oh et al., 2005). As it is evident from Fig. 2, the spectrum specifically at 3350 cm^{-1} was weakened, broadened, and shifted to a higher wavenumber for the ionic liquid treated sample indicated the alteration of hydrogen bonds network in the cellulose structure and lower crystallinity. The same trend was observed for the typical peaks of cellulose and hemicellulose at 1730 cm^{-1}. The results indicated that the ionic liquid treatment was more successful in modifying the lignocellulose structure.

3.6. Effect of pretreatment on substrate morphology

Scanning electron microscopy analysis was conducted to qualitatively assess the surface morphology of the untreated and pretreated materials. As shown in Fig. 3, the dilute acid pretreatment resulted in a modified and destructed morphological structure compared with the untreated wood. However, it was apparent that the major microfibrous in the cell wall structure was not highly affected by the acid treatment. Conversely, a different supramolecular structure was observed for wood treated with [EMIM]OAc. The fibrillar pattern was completely disrupted and a spongier structure, with more conglomerate texture, was formed by the pretreatment. Also, the surface of the ionic liquid treated wood appeared to be disordered.

3.7. Effect of pretreatment on substrate enzymatic digestibility

The untreated and different pretreated substrates along with pure cellulose (Avicel PH-101) as a reference were subjected to enzymatic hydrolysis, and a summary of the results are presented in Table 5. Without pretreatment, no appreciable amount of glucose was produced from the wood even after 72 h of hydrolysis. However, digestibility of the lignocellulose was efficiently improved by both dilute acid and ionic liquid pretreatments. The cellulose conversion progressed quickly during the first 24 h, slowed until 48 h,

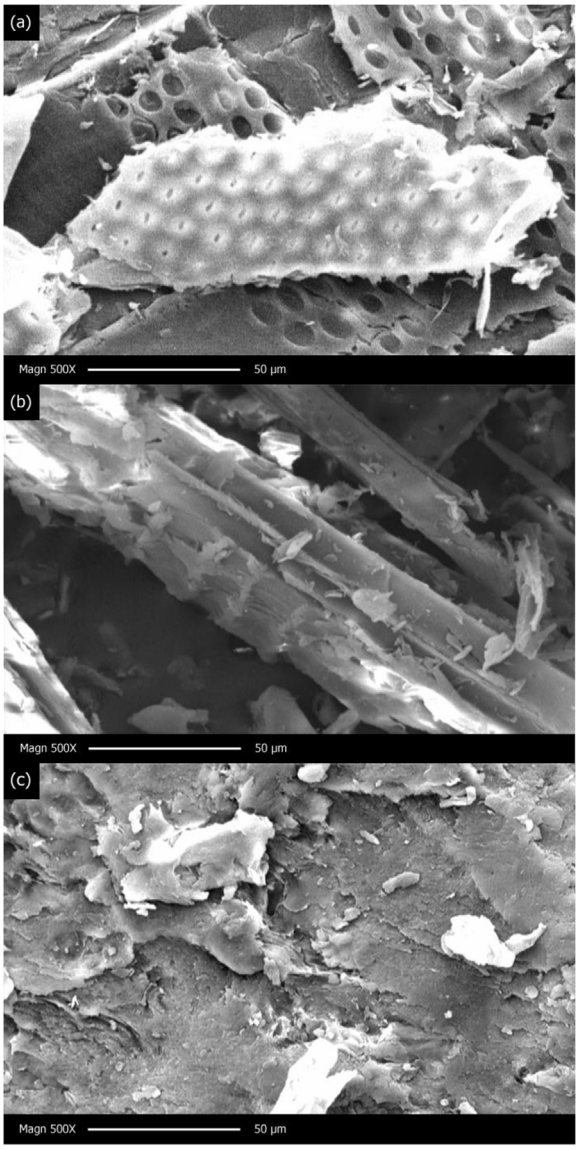

Fig. 3. Scanning electron micrographs of (a) untreated, (b) dilute acid, and (c) [EMIM]OAc (3 h) pretreated aspen wood.

Table 5
Enzymatic digestibility of untreated and pretreated materials.[a]

Substrate	Enzymatic digestibility[b] (%)			
	6 h	24 h	48 h	72 h
Aspen wood				
Untreated	4.4 ± 0.1	5.3 ± 0.2	5.2 ± 0.2	5.3 ± 0.2
Dilute acid treated	27.2 ± 0.8	52.5 ± 0.6	56.1 ± 1.1	59.7 ± 1.9
[EMIM]OAc treated (1 h)	40.2 ± 0.5	78.3 ± 1.0	82.5 ± 2.0	82.1 ± 1.7
[EMIM]OAc treated (3 h)	44.4 ± 0.7	86.0 ± 1.1	89.8 ± 1.6	91.3 ± 1.3
[EMIM]OAc treated (5 h)	49.9 ± 1.1	87.3 ± 0.9	94.3 ± 1.5	95.4 ± 1.0
Pure cellulose	26.4 ± 0.3	45.2 ± 1.1	51.2 ± 1.4	53.3 ± 0.8

[a] The data are average values of the two replications.
[b] The yield was calculated based on grams of glucose released after enzymatic hydrolysis per gram of glucose that can theoretically be produced from glucan in the substrate.

and then remained almost constant up to 72 h. The glucose yield from the untreated wood after 72 h of hydrolysis was only 5.3% of the theoretical glucose yield, while the glucose released from the pretreated wood during the first 24 h of hydrolysis ranged between 52.5% and 87.3% (Table 5). The maximum conversion yield for dilute acid and ionic liquid treated materials were 59.7% and 82.1–95.4%, respectively. Pretreatment of aspen wood with ionic liquid greatly enhanced the rate of hydrolysis and 40.2–49.9% of the cellulose in the ionic liquid treated wood was converted to glucose in just 6 h, versus 26.4% yield when the pure cellulose was hydrolyzed. Compared to the dilute acid pretreatment, the ionic liquid treatment was much more efficient in improving the yield and rate of hydrolysis. The aspen wood after 5 h ionic liquid treatment was the easiest digestible substrate with more than 95% cellulose conversion yield.

3.8. Effect of pretreatment on fermentability of the substrate

Fermentability of the hydrolysates was evaluated using *S. cerevisiae* which is the most important microorganism for industrial ethanol production. This strain was able to produce ethanol with theoretical ethanol yield of 87 ± 3% from glucose and a full supplemented medium. The produced ethanol from the untreated aspen wood was only 5.3% of theoretical ethanol yield after 24 h fermentation, while it was significantly higher for the pretreated materials with a 6.9–14.2-fold increase in the yield (Fig. 4). Only 42.1% of the glucan within the acid treated wood was converted to ethanol. The wood samples treated with the ionic liquid were fermented quicker than the untreated and dilute acid treated wood samples. The percentage of theoretical ethanol yield reached after 24 h fermentation, was 73.5%, 78.9%, and 81.2% for 1, 3, and 5 h ionic liquid treated wood, respectively.

3.9. Mass balance

A summary of the overall mass balance for the different pretreatment, enzymatic hydrolysis, and fermentation stages are presented in Table 6. Recovery of the materials after pretreatment calculated as the oven-dry mass of remained solids divided by initial oven-dried mass. The substrates were recovered at 59.5% and 76.2–81.3% after dilute acid and ionic liquid pretreatments, respectively. The maximum recovery of the materials was observed after 1 h pretreatment by the ionic liquid, whereas the highest amount of ethanol was obtained from the substrate pretreated for 5 h. Approximately, 224 g ethanol produced from 1 kg of the initial aspen wood pretreated with ionic liquid at 120 °C for 5 h, while it was only 15 and 80 g ethanol per kg of the untreated and dilute acid pretreated wood, respectively.

4. Discussion

Growing studies have focused on pretreatment of lignocelluloses such as wheat straw, switchgrass, corn stover, and spruce using different ionic liquids (Poornejad, Karimi, & Behzad, 2013; Sant'Ana da Silva et al., 2011; Shafiei, Zilouei, Zamani, Taherzadeh, & Karimi, 2013; Vancov, Alston, Brown, & McIntosh, 2012). However, there is limited knowledge available regarding the impact of pretreatment on the key features of substrates that promote hydrolytic susceptibility. The present study was conducted to determine the effects of ionic liquid [EMIM][OAc] and dilute

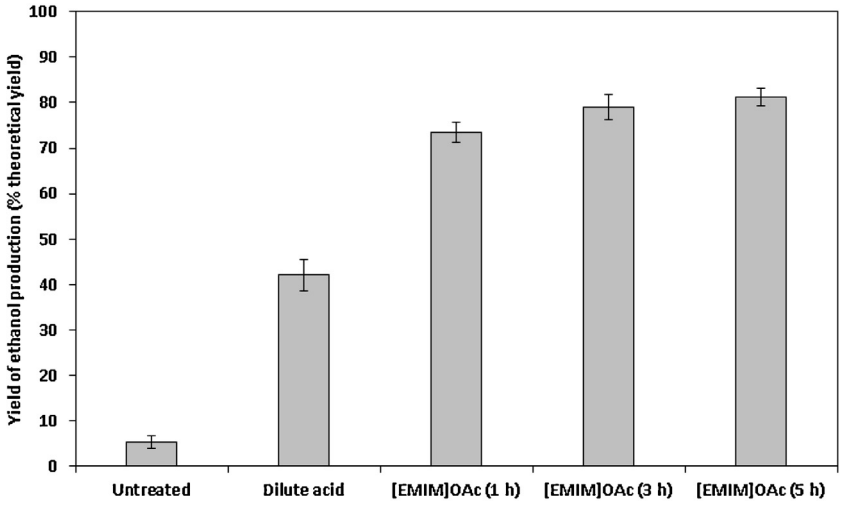

Fig. 4. Yield of ethanol production (percentage of theoretical yield) after 24 h fermentation by *S. cerevisiae*.

Table 6
Summary of the overall mass balance for different pretreatments, enzymatic hydrolysis, and fermentation stages.[a]

	Untreated	Pretreatment			
		Dilute acid	[EMIM]OAc (1 h)	[EMIM]OAc (3 h)	[EMIM]OAc (5 h)
Solid recovery of pretreatment	–	59.5	81.3	80.0	76.2
Produced glucose by hydrolysis[b]	2.8–2.9	19.5–20.8	41.9–44.2	46.2–48.2	47.2–51.0
Produced ethanol[c]	1.5	8.0	20.1	21.6	22.4

[a] All the reported values are grams per 100 g of initial aspen wood.
[b] The ranges are the results of 24 and 48 h hydrolyses.
[c] The data are the results of 24 h fermentation by 5 g/l *S. cerevisiae*.

acid pretreatments on physiochemical properties of aspen wood using semi-quantitative techniques. The pretreated materials were comprehensively compared using their composition, surface area, accessibility to cellulase, enzymatic digestibility, structural and morphological features.

Major improvement in enzymatic digestibility (~18-fold) of aspen wood was observed via the ionic liquid pretreatment and more than 95% of the glucose theoretical yield was achieved. Consequently, the pretreatment with [EMIM]OAc succeeded in improving the fermentability of aspen wood up to about 82% of ethanol theoretical yield after 24 h fermentation using *S. cerevisiae*. For rice straw, poplar wood, and spruce up to 88%, 92%, and 73% hydrolysis yield were reported by [EMIM]OAc pretreatment, respectively (Maki-Arvela, Anugwom, Virtanen, Sjoholm, & Mikkola, 2010; Nguyen et al., 2010; Shafiei et al., 2013).

The regenerated materials had higher level of glucan and lower levels of residual lignin compared to the untreated and dilute acid treated solids. It is acknowledged that the lignin associated with microfibrils occupy the internal space of lignocellulose and restrict the accessibility to hydrolytic enzymes. The delignification effect of ionic liquids has also been investigated for various materials such as spruce and maple woods. For example, more than 32% and 19% lignin removal from maple wood has been observed after acetate containing ionic liquid and [BMIM][MeSO4] pretreatments, respectively (Lee, Doherty, Linhardt, & Dordick, 2009). The difference in the reported values could be due to the specific interactions between solvent molecules and biomass which is directly related to the type of ions in the ionic liquid, biomass recalcitrance, and applied pretreatment conditions.

Analysis of the substrate by Simons' stain and WRV methods showed significant increase in dye adsorption capacity, adsorption ratio, and swelling ability of the ionic liquid treated materials. Direct dyes (e.g. Simons' stain dyes) have high affinities for cellulose hydroxyl groups and can be considered as molecular sensors for cellulose-containing materials. They can be assumed to dock onto the cellulose surface with their long axis parallel to and aligned with the polymer chains (Inglesby & Zeronian, 2002; Wiman et al., 2012). The two direct dyes used in the Simons' stain method are different in molecular size and binding affinity for lignocelluloses, thus distributing in the pores. The small DB (Direct Blue 1) molecules are able to penetrate into both small and large pores whereas the large DO (Direct Orange 15) molecules only diffuse into the larger pores. However, in competition, DO can easily displace the DB from the larger pores due to the stronger affinity with the hydroxyl groups (Esteghlalian et al., 2001). Noticeable increases in dye adsorption capacity (1.3–2.2-fold) and adsorption ratio (1.4–2.9-fold) after the ionic liquid pretreatment indicated major improvement in total surface area (external and internal), pore sizes, and distributions. The diameter of the DO molecules is quite similar to the diameter of the catalytic core domain of endoglucanase from *T. reesei*, and thereby a higher adsorption ratio implies that the expanded pores are large enough to accommodate the enzyme molecules. Moreover, increasing the WRVs of the samples after the ionic liquid pretreatment showed enhanced porosity (internal surface area) of the substrate (Chandra et al., 2009). It should be noted that the addition of anti-solvent (i.e., water) to ionic solvent-biomass solution, make the system thermodynamically unstable, thus rapid phase separation occurs. Nonsolvent-induced precipitation process is believed to happen via the mechanism of spinodal decomposition which leads to finely porous structures (Lu, Yan, & Texter, 2013; Machado, Habert, & Borges, 1999; Peng et al., 2012; Xing, Peng, & Chung, 2011). It could be a possible explanation for enhanced surface area caused by ionic liquid treatment. In the case of dilute acid pretreatment, deposition and accumulation of the hydrophobic lignin on the fibers surfaces might occur and prevent water molecules from reaching the porous regions by occluding the pores structure (Donohoe, Decker, Tucker, Himmel, & Vinzant, 2008). However, more investigation is needed to support this hypothesis.

Substrate accessibility to cellulases plays an important role in enzymatic hydrolysis. Higher surface area and enlarged pores are effective parameters when they can enhance the fiber accessibility to the hydrolytic enzymes. The ionic liquid was capable of great increase in the substrate accessibility to cellulase, whereas the regenerated biomass exhibited higher binding capacity and affinity for cellulase compared to the untreated and acid treated materials. A possible explanation is dissolution of restricted cellulose within the biomass matrix and precipitation of the regenerated form on the biomass surface that provides more accessibility to the cellulose (Li et al., 2009). It should be noted that the enzyme adsorption experiments were conducted at 4 °C to prevent possible hydrolysis of the cellulosic fibers. However, adsorption process is known to be temperature dependent, possibly due to the greater binding constant at lower temperature. Therefore, the lower the temperature, the more protein adsorbs on the surface (Ooshima, Sakata, & Harano, 1983). On the other hand, at higher temperatures (e.g., 10–50 °C) hydrolysis of the fibers could impact the adsorption process by decreasing the active adsorption sites and altering the substrate features.

Recently, impact of ionic liquid pretreatment conditions on cellulose crystalline structure has been investigated for different feedstocks, including microcrystalline cellulose (Avicel) and lignocelluloses (Cheng et al., 2011; Maki-Arvela et al., 2010; Vancov et al., 2012). It has been shown that the pretreatment resulted in a loss of native cellulose crystalline structure. For Avicel, cellulose I lattice expanded and distorted prior to full dissolution in [EMIM][OAc], and upon precipitation the former structure was convert to a less ordered intermediate form, whereas fully dissolved cellulose was regenerated to a mixture of cellulose II and amorphous cellulose. However, for lignocelluloses, ionic liquid mainly disrupted the crystalline structure of cellulose and the pretreated materials exhibited typically lower degrees of crystallinity. In the current study, structural analysis of the materials was examined by FTIR spectroscopy and the ionic liquid treated materials were found to be less crystalline compared to the untreated and acid treated biomass. This could be due to the rapid precipitation during the regeneration step which prevents the dissolved cellulose recombine into the original crystalline structure (Li et al., 2009). Higher LOI value for dilute acid treated wood implied that hot dilute acid may promote some

recrystallization. It might be explained that hydrothermal conditions and partial hemicellulose removal (Table 1) could lead to annealing of cellulose and increase its crystallinity (Karimi, Shafiei, & Kumar, 2013; Li et al., 2010).

In accordance with the results of compositional analysis, the delignification effect of the ionic liquid treatment was well detected from the spectrum. Furthermore, SEM images confirmed that significant morphological modification occurred after the ionic liquid pretreatment compared to the dilute acid treatment. It is expected that the amorphous, porous, and fragmental regenerated cellulose could provide more accessible surfaces area and consequently be more amiable to the enzymatic attack.

The agreement between the digestibility and semi-quantitative analysis results implied that the applied techniques are strong indicators of the changes in substrate and enzyme–substrate factors caused by the ionic liquid and dilute acid pretreatments.

5. Conclusions

Assessment of the ionic liquid and dilute sulfuric acid pretreated aspen wood by rapid semi-quantitative methods showed that the treatment with [EMIM]OAc, even for a short time, resulted in significantly higher accessibility and affinity to cellulase enzyme, lower cellulose crystallinity, and improved morphological features. The aforementioned modifications caused by the ionic liquid pretreatment led to efficient digestibility of aspen wood, whereas the dilute acid was more suitable for xylan removal.

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