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Improved biological delignification of wood biomass via Ionic liquids pretreatment: A one step process

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Abstract

The enzymatic pretreatment of wood biomass for degrading lignin, a complex aromatic polymer, has received much attention as an environmentally safe or "green" process. However, this process for lignin degradation has been found to be very slow, even needed several months. To overcome this limitation, this study reports a new approach for enhanced enzymatic delignification of wood biomass using room temperature ionic liquids (RTILs)- a potentially attractive "green" and "designer" solvent- as (co)solvents or/and pretreated agents. The method comprised pretreatment of wood biomass prior to enzymatic delignification in ILs-aqueous systems with the aim of overcoming low delignification efficiency associated with the difficulties in enzyme accessibility to the solid substrate and the poor substrate and products solubility in aqueous system. The results showed that IL [emim] [OAc] (1-ethyl-3-methylimidazolium acetate) was better solvent for wood delignification than IL [bmim][Cl] (1-butyl-3-methylimidazolium chloride). The recovered cellulose rich materials obtained from combination effects of IL and biological pretreatment contained significantly lower amounts of lignin as compared to the amounts found when each method applied alone. The produced cellulose rich materials were characterized by acid hydrolysis, Fourier-transform infrared spectroscopy (FTIR), Scanning electron microscopy (SEM), and X-ray diffractometry (XRD). SEM and XRD revealed considerable microstructural and crystallinity index changes in the pretreated cellulose rich materials. We believe that this newly developed process will play a great role in converting cellulosic biomass- the most abundant renewable biomaterials in the world- to biomaterials, biopolymers, biofuels, bioplastics and hydrocarbons.

Keywords: ionic liquids, wood biomass, cellulose, lignin, laccase, enzymatic delignification.

1. Introduction

The rapidly growing demand for energy, uncertainty about the costs and supply of petroleum and concerns about environmental impact by the use of petroleum based resources have led to motivated interest in alternative resources, particularly from renewable resources including lignicellulosic biomass. Considering these facts, the use of lignocellulosic based materials in various sectors (*e.g.,* automotive and aerospace) over petro- materials has received increased attentions due to the growing global environmental awareness and concepts of sustainability and industrial ecology and no conflict between food *vs* materials. Wood – the most abundant lignocellulosic resources on the world – consists of up to 50% cellulose that is rigid semi-crystalline embedded in amorphous hemicelluloses and lignin. Cellulose and lignin –Earth's most and second most abundant biopolymer, respectively– represent an enormous carbon-neutral renewable resource for biomaterials and bioenergy production**.** This is why, the sepapartion of such components from wood biomass has gained a great deal of recent interest. However, the recalcitrant nature of the wood cell wall represents the biggest challenge in the development of wood biomass to biomaterials/ biocomposites technologies. In fact, a distinct crystalline structure of cellulose makes it a challenge to find suitable solvents for its dissolution as well as isolation from lignin. To date, a number of pretreatment approaches including physical (*e.g.,* pyrolysis and mechanical disruption)(Moiser et al., 2005), physico-chemical (*e.g.,* steam explosion and ammonia fiber explosion (Hendricks & Zeerman, 2009), chemical (*e.g.,* acid hydrolysis, alkaline hydrolysis and oxidative delignification) (Merino et al., 2007), and biological methods (Lee, 1997; Bak et al., 2009) have been investigated to delignify wood biomass for extraction of cellulose. Many of these methods require high temperatures and pressures, as well as highly concentrated chemicals, for the cooking process. Conventional chemicals, such as sulfates, and

sulfite pulping processes pose serious environmental hazards in air and water. Moreover, high temperature based cooking processes result in the production of inhibitory chemicals and degradation products. On the other hand, the biological pretreatment for wood delignification is an environmentally safe. Generally, enzymes isolated from naturally occurring fungi, or with enzymes produced by genetically engineered fungi have been used for wood biodegradation. However, this approach in aqueous system has been found to be very slow mainly due to the difficulties in enzyme accessibility to the solid substrate and the poor solubility of lignin (Martinez et al., 2009). It is therefore desirable to develop a biomass pretreatment process that is not only the environmentally friendly but also efficient and cost effective for biomass conversion to cellulose and lignin.

The poor solubility of substrate and products during wood delignification in aqueous systems can be overcome by using ionic liquids (ILs) as cosolvents. It is well recognized that ILs, a potentially attractive "green" recyclable alternative to environmentally harmful organic solvents, have been increasingly exploited as solvents and/or (co)solvents and/or reagents in a wide range of applications including pretreatment of lingo-cellulosic biomass (Kilpelainen et al., 2007; Mora-pale et al., 2011; Sun et al., 2009). The very high solvating properties of ILs have been exploited in the dissolution of cellulose (Swatloski et al., 2002), lignin (Pu, 2007) and wood (Sun et al., 2011). Generally, hydrophilic ILs are able to completely solubilize wood at over 100 ºC, and cellulose rich materials can readily be precipitated with an anti-solvent, such as acetone and ethanol. The degree of polymerization (DP) of regenerated cellulose was found to be reduced notably which lead to enhanced enzymatic cellulose hydrolysis, making the system suitable for biomass to biofuels technologies (Lee et al., 2009). However, the production of high strength biomaterials and biocomposites requires structurally strong cellulose fibers. It is worthy to mention that less attention was paid on designing and development of IL-based technology which can extract cellulose with minimum altered structure from wood.

Unfortunately, the practical obstacle of using ILs for enzymatic delignification is that many ILs, particularly hydrophilic ones have negative effect on enzyme structure, resulting in deactivation of enzyme (Moniruzzaman et al., 2008, 2009, 2010a, 2010b). Such effects could be balanced with the increase the solubility of substrates and products leading to better performances in terms of enhanced yield. Recently, it was reported that laccases can maintain their activity for the oxidation of 2, 2′-azino-bis(3-ethyl-benzthiazoline-6-sulfonic acid) and catechol in ILs-water systems containing over 80% of water (Shipovskov et al., 2008;Tavares et al., 2008). This is consistent what was reported for the performances of others enzymes in IL alone or IL-water systems (Moniruzzaman et al., 2010 a).

To this end, recently, it was found that enzymatic delignification efficiency can be improved by IL pretreatment of wood biomass prior to enzymatic delignification in aqueous systems in the presence of small amount of water (Moniruzzaman & Ono, 2012). In this one step process, 10 wt% wood chips in an IL were cooked and then aqueous solution containing enzyme was added directly to start the delignification. Preliminary results indicated that enzymatic delignification efficiency of IL-swollen wood biomass became higher than that of untreated materials. In fact, wood biomass is swollen by ILs prior to delignification provided increased surface area accessible to the enzymes. The system has a significant advantage because the substrate and product solubility are expected to increase in ILs which may enhance the process efficiency. This notble finding inspires us to investigate how the major process parameters such as type of ILs and cooking time affect delignification. We believe that delignification efficiency will be improved to a extent after optimization such parameters.

Here, the objective of this study is to comduct enzymatic delignification of wood biomass pretreated with ILs using laccase as a biocatalyst. The goal of pretreatment is to with the aim of overcoming low delignification efficiency associated with the difficulties in enzyme accessibility to the solid substrate and the poor substrate and products solubility in aqueous system.The effect of the major parameters including types of ILs and incubation time in ILs were investigated. The treated wood fibers were characterized using Fourier-transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM), and X-ray diffractometry (XRD) and compared those with untreated wood fibers. Commercial laccase which is a copper-containing oxidase enzyme obtained from white rot fungi, was selected as a biocatalyst because it can degrade the lignin of biomass leaving the other components (*e.g.,* cellulose) virtually untouched (Blanchette, 1991).

2. Materials and method

2.1 Materials

Wood chips from hinoki cypress (*Chamaecyparis obtusa*) were received from Okayama Biomass Center, Japan. Alkali lignin and 1-Hydroxybenzotriazole (HBT) were purchased from Aldrich Chemical Co. (St. Louis, MO). The IL [emim][OAc](1-ethyl-3-methylimidazolium) (≥ 95%) and IL [bmim][Cl] (1-butyl-3- methylimidazolium chloride) were obtained from Ionic Liquids Technologies GmbH (Heilbronn,Germany) and used as received. 2,20-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) (98%) was obtained from Sigma (St. Louis, MO). Commercial Laccase Y120 (EC. 1.10.3.2) (1000U/g) from Trametes *sp.* was kindly supplied by Amano Enzyme Inc. (Nagoya, Japan).

2.1 Process

A simplified overview of experimental method was shown in Figure 1. Firstly, wood chips were grounded into powders through a lab-scale roller mill and passed through sieves to separate fractions with 110–550 µm particle sizes which were dried overnight in an oven at 110 °C. In a typically experiment, 200 mg of wood were added to 2 g IL in a three neck flask and heated at 80 °C in an oil bath with magnetic stirring for desired time. After cooling the wood–IL mixture to room temperature, acetate buffer (100 mM, pH 4.5) containing laccase were added to the flask, whereas 1-hydroxybenzotriazole (HBT) (1.5 wt% of wood chips) was added as a mediator. Reaction was carried out with the supply of O_2 bubbles with a small stirrer bar at 50 °C. After cooling the reaction mixture to room temperature, 0.1 M NaOH was used to wash ILs and lignin away from the cellulosic fibers. To remove traces of NaOH, the fibers were washed with distilled water until pH paper showing the final drops of washing liquid to be pH neutral. The lignin content in the filtrate NaOH solution was determined by measuring absorbance at 280 nm (Kilpelainen et al., 2007). Alkali lignin from Aldrich Inc. was used to prepare the calibration curve (see Figure 2). After drying the treated wood fibers in a convection oven at 65 °C for 48 h, sample was weighted and stored at vacuum desiccator. The recovery of IL for further use was carried out as described previously (Tan et al., 2009). The content of untreated wood was determined using TAPPI methods with a scaled down process.

2.2 Enzyme assay

Laccase activity was determined by oxidation of 2-2'.azmobis-(3.ethyl benzthiazoline-6-sulphonate) (ABTS). The reaction mixture contained 0.5 mM ABTS, 0.1 M sodium acetate buffer, pH 5.0, and a suitable amount of enzyme. Oxidation of ABTS was followed by absorbance increase at 420 nm (ε_{420} = 3.6 x 10^4 M^{-1} cm^{-1}). One unit was defined as the amount of enzyme that oxidized 1 µmol of ABTS per min and the activities were expressed in U/gm.

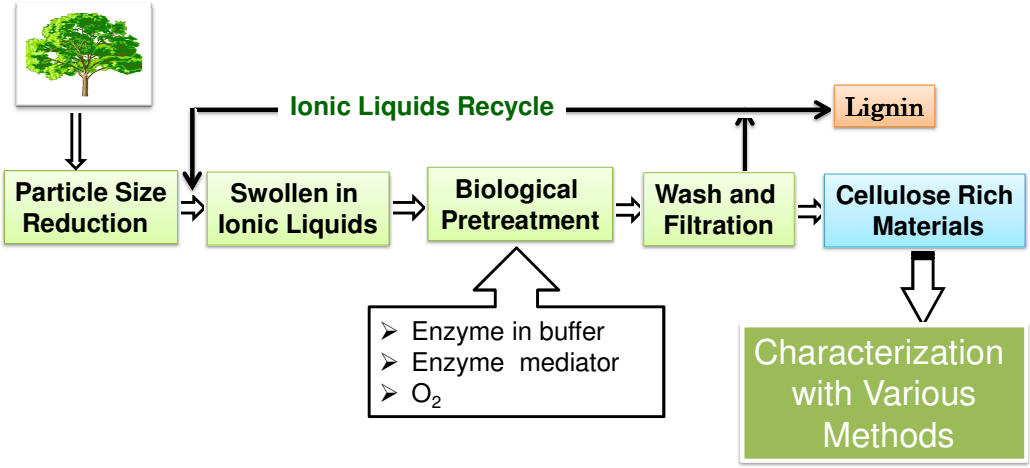

Figure 1. Flowchart of the enzymatic delignification of wood biomass using ionic liquids as pretreatment agents and cosolvents.

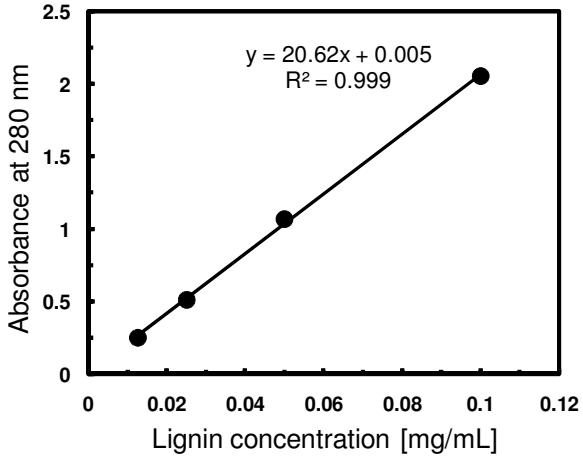

Figure 2. Calibration curve of alkali lignin dissolved 0.1 M NaOH containing 0.2 wt% IL [emim][OAc]

2.3 Characterization of treated and untreated wood materials

2.3.1 Morphology of materials

The fibers morphology was characterized using a scanning electron microscope (SEM) (S-4700, Hitachi Ltd., Tokyo, Japan). For SEM images, fibers were mounted on metal stubs by double- faced tape and images were taken. Prior to imaging samples were coated with gold–palladium in a sputter coater (E1030 Ion Sputter, Hitachi Ltd.).

2.3.2 Fourier transform infrared spectroscopy (FTIR)

The FTIR spectra of the samples were recorded from a KBr disk containing 1% finely ground samples on an IRPrestise-21 FTIR spectrophotometer (Shimadzu, Japan) in the range of 4000–400 cm^{-1} with a resolution of 4 cm^{-1}. Spectral outputs were recorded in the transmittance mode as a function of wave number.

2.3.3 Powder X-ray diffraction (PXRD)

The crystallinity of the untreated and treated wood materials was investigated by powder X-ray diffractometry (PXRD), using a XRD-6100 Diffraction System (Shimadzu, Japan). The diffraction patterns were measured from $2\theta = 8–40°$ with scan speed of $0.1° \text{ min}^{-1}$ using Cu Kα radiation at 40 kV and 30 mA.

3. Results and discussion

3.1 Ionic liquid pretreatment of wood biomass

Pretreatment of wood biomass (10 wt%) with IL [bmim][Cl] and [emim][OAc] was carried out at moderate conditions (80 °C, 1-3 hrs) to swell the wood cell by partial dissolution. Here, we have selected these two ILs because they are able to dissolve wood materials at higher temperatures (Kilpelainen et al., 2007; Sun et al., 2009). In general, temperatures from 80 to 130 °C have been used to dissolve wood materials in ILs (Kilpelainen et al., 2007; Fort et al., 2007). Although, elevated temperatures (100 °C or higher) lead to complete dissolution of wood biomass which favors delignification efficiency, the crystallinity of regenerated cellulose rich materials decreased and loss of biopolymer increased significantly (Labbe et al., 2012; Lucas et. 2011; Wang et al., 2011; Weerachanchai et al., 2012). For this work, we have selected moderate temperature during IL pretreatment so that the major components of wood particularly, cellulose loss were minimized. In addition, cellulose can also be extracted with minimum structural alteration. After completing pretreatement at 80 °C under vigorous mechanical stirring, the colors of the mixture became dark and their viscosities increased, indicating that patial dissolution of wood occurred. Then, the mixture was diluted with acetate buffer and enzymatic delignification was conducted as stated in the experimental section.

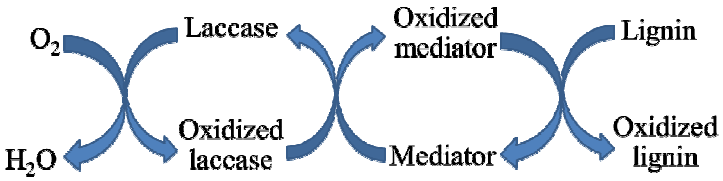

Figure 3. Catalytic cycle of a laccase-mediator lignin degradation system.

3.2 *Enzymatic delignification of IL treated and untreated wood biomass*

Enzymatic delignification of IL treated wood biomass was investigated using two type of ILs. Catalytic cycle of a laccase-mediator lignin degradation system is shown is Figure 3. The results obtained from enzymatic delignification in IL alone and IL-aqueous systems are shown in Table 1. For comparison, enzymatic delignification in aqueous systems and delignification in ILpretreatment were also performed. The results clearly demonstrated that enzymatic delignification of wood biomass swollen by IL prior to enzymatic delignification could be an efficient method for the removal of lignin to extract cellulose fibers. Comapred to IL [bmim][Cl], IL [emim][OAc] was found to be suitable for pretaement of wood biomass and enzymatic deliglification (entries 3&5), possibly, due to its high ability in dissolution of wood (Sun et al., 2009), and its enzyme compatible nature (Zhao et al., 2008). Another possible reason of lower delignification efficiency using IL [bmim][Cl] is well known adverse effect of Cl^- on enzyme performace (Lee et al., 2006). It is clearly indicated from Table 1 that IL pretreatment did not significantly change the lignin composition of wood materials but did alter the structure to render a more accessible surface area for enzyme. Since IL [emim][OAc] gave the best results, this IL will be used for subsequent experiments. The enhanced process efficiency with IL [emim][OAc] may be a combination of factors. First, the swollen of ground wood may increase the available surface area to the enzymes. In addition, ILs can dissolve some lignin during swollen, which can lead to increase the enzyme accessibility. Note that IL [emim][OAc] was found to be selective for lignin during pretreatment of wood biomass (Lee et al., 2009) Second, the substrate and product solubility are expected to increase by using ILs, which are responsible for low delignification efficiency in aqueous system.

Table 1. Lignin extraction from wood biomass with different methods

entry	Ionic liquid used for pretreatment[a]	Reaction media for enzymatic delignification	Extracted lignin[b]
1	No IL pretreatment	Enzymatic delignification in acetic buffer [c]	10.2
2	No IL pretreatment	5% (w/w) IL[bmim][Cl] in buffer	3.1
3	No IL pretreatment	5% (w/w) IL[emim][OAc] in buffer	16.5
4	[bmim][Cl]	5% (w/w) IL[bmim][Cl] in buffer [d]	14.2
5	[emim][OAc]	5% (w/w) IL [emim][OAc] in buffer [d]	48.4
6	[emim][OAc]	No enzymatic delignification [e]	7.0

[a] 200 mg of ground wood were incubated in 2g IL at 80ºC with vigorous magnetic stirring for 1hr

[b] results are expressed as a percentage of extracted lignin relative to lignin content in the original ground wood. The data are the average of three experiments.

[c] reaction conditions: 200 mg untreated wood, 10 mL of 100 mM sodium acetate buffer (pH = 4.5), 50 U laccase, 50°C, 24 hr and 1-hydroxybenzotriazole (HBT) = 3 mg.

[d] 200 mg wood, buffer 38 mL, 23 hr and other reaction conditions are the same as for entry 1;

[e] 200 mg wood swollen by 2 g IL, buffer 38 mL, 50°C and 23 hr.

3.3 Effect of pretreatment time

To understand the correlation of wood biomass enzymatic delignification with IL pretreatment time, various samples of IL[emim][OAc]-pretreated wood biomass were prepared by changing the treatment time in the IL.

Generally, long cooking time increases the delignification efficiency; however use of energy becomes very important at long cooking time. The incubation time for pretreatment of wood in in IL was varied from 0.5 to 3 h at 80 °C (see Figure 4). It was found that delignification efficiency increased with increasing pretreatment time. For example, pretreatment time from 0.5 to 3 hr, the delignification efficiency for wood biomass increased from 24.1 to 64.8%. This result is consistent what have been reported in the literature (Lee et al., 2009). One possible explanation is that IL pretreatment of wood biomass can easily swell cell walls to weaken the network of biomass components, which leads to dissolution of wood in IL with pretreated time (Lee et al., 2009). Consequently, lignin extraction was promoted due to the dissolution of biomass with iincubation time. However, the crystallinity of recovered cellulose rich fibers decreases with the increase in preatreatment time as shown in Figure 5. Since, our objective is to extract cellulose fibers with minimum structural alteration, we used ptratement time 1 hr, as compromise between high delignification and high crytallnity of the cellulose rich materials, for subsequent experiments.

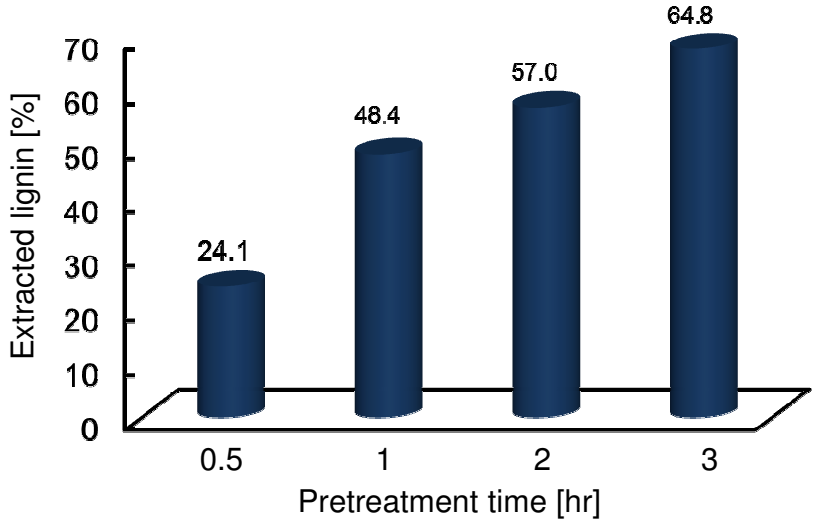

Figure 4. Effect of cooking time in IL [emim][OAc] on delignification of wood biomass. 200 mg of ground wood were incubated in 2g IL at 80ºC with vigorous magnetic stirring. Enzymatic delignification conditions are the same as entry 1 shown in Table 1.

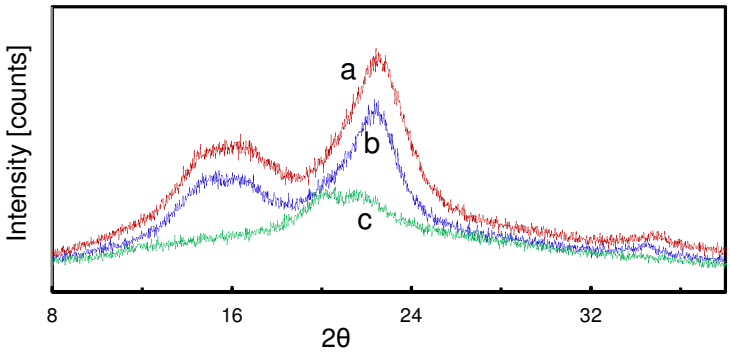

Figure 5. X-ray diffraction spectra of wood biomass pretreated with IL for (a) 1 hr, (b) 2 hr and (c) 3 hr followed by enzymatic delignification.

3.4 Chracterization of treated and untretaed wood materials

Our next aim was to characterize treated and untreated wood fibers using different techniques in order to better understand compositional and structural impacts. As shown in SEM images (Fig. 6), pretreated wood fibers have shown a different morphology compared to untreated wood materials. In cellulose rich materials, wood cell networks composed of cellulose, hemicellulose and lignin were broken down and cellulose fibers were partially

separated into individual microsized fibers (Fig. 6b). Significantly, obtained cellulose rich materials have smooth and clean surfaces (data not shown) because most of the non-cellulosic materials (*e.g.,* lignin) were removed during the IL and enzymatic delignification.

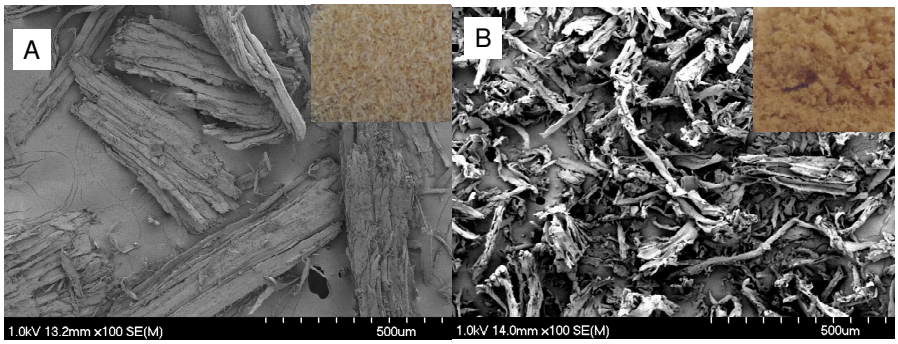

Figure 6. SEM images of (a) Untreated ground wood, and (b) the corresponding enzymatic treated wood fibers (entry 5 in Table 1). Picture of treated and untreated wood biomass is shown in inset.

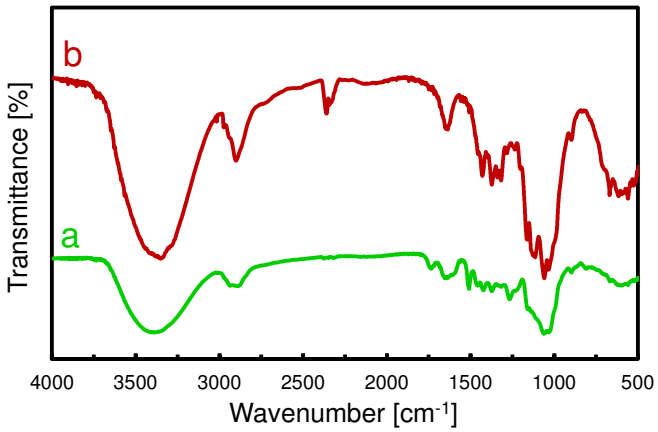

Figure 7. FTIR spectra for (a) untreated wood materials, (b) IL Treated wood fibers followed by enzymatic delignification.

The FTIR spectra of the untreated and treated samples were measured and are compared in Figure 7. The dominant peaks at ca. 3346 cm^{-1} (O-H stretch) and ca. 2892 cm^{-1} (C-H stretch) represent the aliphatic moieties present in major wood material biopolymers. The prominent peak at 1731 cm^{-1} in the untreated wood materials is attributed to a C=O stretching vibration in acetyl groups of the hemicelluloses (Labbe et al., 2005). The characteristic peaks of lignin at 1592/1503 (C=C stretching vibration), 1256 cm^{-1} (asymmetric bending in CH_3), and 1251 cm^{-1} (C-O vibration in the syringyl ring) (Labbe et al., 2005) disappeared after IL pretreatment followed by enzymatic delignification due to the removal of most of the lignin. The absorbance bands at 1150 cm^{-1}, 1052 cm^{-1} and 896 cm^{-1}, corresponding to C-O-C asymmetric bridge stretching vibration in cellulose/hemicellulose, C-O stretching vibration in cellulose/hemicellulose, and C-H deformation vibration in cellulose, respectively, (Labbe et al., 2005), were more resolved in the obtained cellulose rich materials, indicating that the produced cellulose-rich wood fibers are richer in carbohydrates, consistent with our chemical composition study.

4. Conclusions

This study reported an environmentally friendly and efficient approach which was comprised ionic liquid pretreatment followed by enzymatic delignification for isolating cellulose fibers with minimum structural

alteration from wood biomass. IL[emim][OAc] has been found to be a better solvent/agent than [bmim][Cl] for wood pretreatment and enzymatic delignification. Compared to conventional delignification of wood biomass in aqueous system, delignification efficiency was increased significantly for IL treated wood; at optimized condition about 65% delignification was obtained where as it was about 10.2 % without IL pretreatment. This enhanced efficiency was due to the improved solubility of substrates and products in ILs and easy enzyme accessibility to the IL swollen wood cell prior to the delignification. The produced cellulose rich materials were characterized by acid hydrolysis, Fourier-transform infrared spectroscopy (FTIR), Scanning electron microscopy (SEM) and X-ray diffractometry (XRD). SEM and XRD revealed considerable microstructural and crystallinity index changes in the pretreated cellulose rich materials. The combination of IL pretreatment and enzymatic delignification may provide a platform for cellulosic biomass to biomaterials, biopolymers, biofuels, bioplastics and hydrocarbons.

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