Ionic Liquid Pretreatment of Softwood and Herbaceous Materials for Enhancing Enzymatic Saccharification

January 2018

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Ionic Liquid Pretreatment of Softwood and Herbaceous Materials for Enhancing Enzymatic Saccharification

A Dissertation Submitted to

The Graduate School of Life and Environmental Sciences

University of Tsukuba

In Partial Fulfillment of the Requirements

For the Degree of Doctor of Philosophy in Bioresource Engineering

(Doctoral Program in Appropriate Technology and Sciences for Sustainable Development)

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List of Abbreviations

[Bmim]Cl	1-butyl-3-methylimidazolium Chloride
[Emim]Ac	1-ethyl-3-methylimidazolium Acetate
[Emim]Cl	1-ethyl-3-methylimidazolium Chloride
CHN	Carbon Hydrogen Nitrogen
C_rI	Crystallinity Index
DMAC	*N, N*-dimethylacetamide
DMSO	Dimethyl Sulfoxide
ED	Electrochemical Detector
FPU	Filter Paper Unit
IL	Ionic Liquid
ILs	Ionic Liquids
NaOH	Sodium Hydroxide
SEM	Scanning Electron Microscope
SP	Single Pump
UV	Ultraviolet
UV-vis	Ultraviolet-visible
XRD	X-ray Diffraction

Chapter 1 General Introduction

1.1 Introduction to lignocellulosic materials

The current supply of raw materials in terms of fuel, energy, and chemicals, depends on processing crude oil. Limited fossil resources, knowledge of their impacts, and increasing global fossil fuel demand encourages exploration into sustainable resources. The shift of raw materials to chemical processing offers the possibility of innovative products and thus more efficient production lines. The exploitation of biological resources in new processes is sometimes required to develop sustainable processes based on light and selective conversion steps.

The most significant and available renewable carbohydrate is estimated to come from lignocellulosic biomass, which has the potential to generate several billion tonnes per year [1]. One potential source of lignocellulosic biomass is wood. It does not compete with edible plants and thus has the potential to provide raw materials that generate added value from wood. Wood is very resistant to biological, chemical, and mechanical attacks due to the nature of its structure.

Lignocellulosic biomasses such as rice straw, sugarcane bagasse, cotton stalk, bamboo, wheat straw, and sugarcane tops, are abundantly available as agro-residues. The impressive growth rate, lack of land use competition, high cellulose content, and low lignin content of water hyacinth has led it to be a suitable lignocellulosic biomass [2]. Several factors, such as lignin content, particle size, cellulose crystallinity, hemicellulose and cellulose content exist in lignocellulosic biomass. The presence of lignin and hemicellulose inhibit the enzyme to reach cellulose. Pretreatments have a goal to improve the hydrolysis of lignocellulosic biomass [3,4].

The beneficial effects of pretreatment of lignocellulosic materials have been recognized for a long time. The purpose of the pretreatment process is to remove lignin and hemicellulose, reduce cellulose crystallinity, and increase the porosity of lignocellulosic materials. Pretreatment must meet the following requirements: (1) improve the formation of sugars or the ability to subsequently form sugars by hydrolysis, (2) avoid the degradation or loss of carbohydrate, (3) avoid the formation of byproducts that are inhibitory to the subsequent hydrolysis and fermentation processes, and (4) be cost-effective [3,4].

The selective and efficient conversion process for lignocellulosic biomass is very challenging. In 2002, the dissolution of cellulose was discovered in ionic liquids (ILs) at mild temperatures [5]. This presents an outstanding capability, as common solvents do not enable cellulose dissolution and processing in the homogeneous phase. Moreover, ILs are non-volatile, which is in contrast to organic solvents, whose emissions contribute to global warming. In more recent years, the first reports on the dissolution of wood with ILs have been published [6].

1.2 Chemical composition of lignocellulosic materials

Lignocellulose is the main component of plant cell walls. Approximately 90% of the dry weight of most lignocellulosic materials is stored in the form of cellulose, hemicellulose, lignin, and pectin [4]. Wood mainly consists of 40-45 wt % cellulose, 20-30 wt % hemicelluloses, and 26-32% lignin [7]. Grasses consist of 25-40 wt % cellulose, 35-50% wt % hemicellulose, and 10-30 wt% lignin. Plant biomass also composed of smaller amounts of protein, extractives (soluble nonstructural materials such as nonstructural sugars, nitrogenous material, chlorophyll, and waxes), and ash [3,4,7].

1.2.1 Cellulose

A cellulose macromolecule consists of several glucose monomers that are linked to a dehydration reaction, with water as a by-product. The repeating unit of cellulose, the monomer, consists of a 6-membered ring of carbon atoms and one oxygen atom. The precise molecular formula of cellulose is $C_{6n}H_{10n+2}O_{5n+1}$. Cellulose is a homopolysaccharide composed of β-D-glucopyranose units. These are linked together by (1 → 4)-glycosidic bonds. Cellulose molecules are linear and have a strong tendency to form intra and intermolecular hydrogen bonds. Bundles of cellulose molecules are thus aggregated together in the form of microfibrils, in which highly ordered crystalline regions alternate with less ordered amorphous regions. The fibrous structure and strong hydrogen bonding give cellulose a large tensile strength and insolubility in most solvents [7].

The crystalline structure of cellulose has been characterized by X-ray diffraction analysis and by methods based on the absorption of polarized infrared radiation. The unit cell of native cellulose (cellulose I) consists of four glucose residues.

All chains of native cellulose microfibrils are oriented in the same parallel direction. Regenerated cellulose (cellulose II) has antiparallel chains. Cellulose II is formed whenever the lattice of cellulose I is destroyed, i.e., for swelling with strong alkali solvents or on the dissolution of cellulose. Since the strongly hydrogen bonded cellulose II is thermodynamically more stable than cellulose I, it cannot be reconverted into the latter. All naturally occurring cellulose has the structure of cellulose I. Cellulose III and IV are produced when celluloses I and II are subjected to specific chemical treatments and heating [7,8].

1.2.2 Hemicellulose

Another group of carbohydrates found in wood are hemicelluloses. These are also polysaccharides. However, they constitute of hexoses and pentoses, i.e., xylose, mannose, glucose, rhamnose, galactose, and arabinose. They are organized in a chain to form the main backbone, which is frequently branched and substituted with sugars. Hemicelluloses are heteropolysaccharides. Like cellulose, most hemicelluloses function as supporting materials in the cell walls. Hemicellulose is relatively readily hydrolyzed by acids to their monomeric components consisting of D-glucose, D-mannose, D-xylose, L-arabinose, and a small amount of rhamnose, in addition to D-glucuronic acid, 4-*O*-methyl-D-glucuronic acid, and D-galacturonic acid. Hemicellulose usually comprises between 20 and 30% of the dry weight of wood [7].

1.2.3 Lignin

Lignin, the third structural polymer in wood, consists of aromatic moieties with a rather random structure. Lignin is a polymer consisting of phenylpropane units. Guaiacyl lignin, which occurs in almost all softwoods, is mostly a polymerization product of coniferyl alcohol. The guaiacyl-syringyl lignin, typically occurring in hardwoods, is a copolymer of coniferyl and sinapyl alcohol. Many studies have indicated that covalent linkage must exist between lignin and wood polysaccharides. Hemicellulose components (xylan and galactoglucomannans in softwood) are bound to lignin mainly through arabinose, xylose, and galactose moieties [7,9].

In addition to the structural carbohydrates, several non-structural compounds are present in wood, commonly referred to as extractives and inorganic components. Although extractives like fats, waxes, tannins, acids, oils, etc., are existent only in a few percentages (to a greater extent in softwood), they determine the smell, color, and resistance against degradation of wood. Extractives are not a major constituent of wood but might prove to be beneficial sources for specialty chemicals and pharmaceuticals. The inorganic components turn into ash after combustion and consist of calcium, magnesium, potassium, and the like [7].

1.3 Water hyacinth (*Eichhornia crassipes*)

Indonesia has 1 million ha of natural lakes and thousands of hectares of man-made water reservoirs. Some of these are eutrophic, mainly due to human activities, and thus provide suitable habitats for the luxurious growth of aquatic plants. One of the most important species is the water hyacinth, which proliferates and migrates easily. It can infest a large area of water in a relatively short time [10].

Various kinds of biomass with no significant purpose can easily be found in Indonesia if forms such as herbaceous, wood, fruit, or aquatic biomass. Several aquatic weed species growing wildly in water areas were considered as undesirable members of the local ecosystem and were identified as local aquatic pollution. Water hyacinth is one of the main culprits, along with the aquatic weeds *Hydrilla verticillata*, and *Myriophyllum spicatum.* Some lake and ponds in Indonesia have been monitored, and the presence of pollutant aquatic weeds evaluated. For example, water hyacinth grows in extremely high areas of biomass, which form mats at the bottom and surface of Toba lake (the largest lake in tropical Asia, located in the middle of North Sumatra). Once the water hyacinth contaminated biomass is harvested, it becomes aquatic waste. This increases the potential for biomass as a renewable energy resource, without financial demands for its cultivation [11].

Water hyacinth is a perennial, mat-forming, herbaceous monocotyledon member of the pickerelweed family (Pontederiaceae) and is native to Brazil and tropical America. It is also a popular ornamental plant found in water gardens and aquariums and bears beautiful blue to lilac colored flowers, round to oblong shaped leaves, and waxy-coated petioles (**Fig. 1.1**) [12,13].

The mature water hyacinth plant consists of roots, rhizomes, stolons, leaves, inflorescences, and fruit clusters. The roots are fibrous, unbranched, and with a

conspicuous root cap. They turn purplish in exposed situations but are white when in darkness or when rooted in soil. They vary little in diameter but considerably in length (0.3 ft. to 3.0 ft. or possibly more) [13]. The stems and leaves contain air-filled bags, which help them stay afloat. Water hyacinth is considered a dangerous weed in many parts of the world because it proliferates quickly and depletes the nutrient and oxygen contents in the water [12, 14].

Table 1.1 shows the carbohydrate and lignin contents of water hyacinth from different sources. Evaluation of the composition of water hyacinth is essential due to the variation in values of carbohydrate and lignin contents in different studies. Biotic and abiotic factors, such as differences in species, growth state, and harvesting time, influenced the carbohydrate and lignin contents. Even though the lignin content of water hyacinth shows varying values, it is still lower than other lignocellulosic materials, which theoretically renders water hyacinth suitable for the pretreatment process [2].

Fig. 1.1 Water hyacinth in water body

Table 1.1 Composition of water hyacinth (carbohydrates and lignin)

Water hyacinth fractions	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Reference
Whole plant	18.1	28.2	7.0	(Zhang et al. 2016) [15]
Stems-leaves	35.2	19.7	15.3	(Das et al. 2014) [16]
Whole plant	16.4	42.8	5.5	(Rezania, Din, and Taib 2017) [17]
Whole plant	18.3	23.3	17.7	(Gao et al. 2013) [18]
Whole plant	35.8	19.4	13.2	(Manivannan and Narendhirakannan 2015) [19]
Leaves	33.8	26.8	14.2	(Das et al. 2014) [20]
Stems-leaves	19.2	40.0	4.8	(Singh and Bishnoi 2013) [21]

1.4 Pretreatment with ILs

There are a number of biological, chemical and physical technologies available for the pretreatment of lignocellulosic biomass, such as the use of an enzyme, ball milling, steam explosion, acid, alkali, lime, and wet oxidation. Environmental concerns and the production of inhibitors to fermenting yeasts during these pretreatment processes need to be considered [22]. **Table 1.2** displays the advantages and disadvantages of using some of these popular pretreatments. There is no perfect pretreatment method, but ILs are interesting because they have an outstanding ability to pretreat lignocellulosic materials at relatively low temperatures and are more environmentally friendly.

ILs are liquids consisting of ions. A liquid composed of ions at approximately room temperature is generally called room temperature ILs, liquid salt of room temperature, or organic ion liquids. The interparticle force that occurs in ILs is the coulombic force between anions and cations. Theoretically, there are billions of types of ILs. ILs differ from organic solvents because they cannot evaporate into gas. Therefore, ILs are safe from causing new air pollutants or harmful gases in experiments. Theoretically, ILs can also be recycled several times [23].

In general, ILs have very high viscosities, which thus limits their use as solvents (for example, alkyl imidazolium chloride). Despite this, some ILs have relatively low viscosities. There are several criteria to think about when choosing ILs for big scale operations. The most important ones are costs, physical properties, toxicity, corrosivity, availability, water tolerance, and biodegradability. For examples, [Bmim]Cl is a suitable solvent for cellulose, exhibits relatively moderate toxicity compared with [Emim]Cl. A more efficient alternative to chloride-containing ILs is [Emim]Ac, which is also considered biodegradable, and reasonably non-toxic and non-corrosive [24].

ILs can dissolve organic, inorganic, and polymeric substances. ILs are good solvents in many chemical reactions. The solubility of ILs is closely related to the cationic and anionic properties. Alteration of the alkyl cation will change the dissolution capability of ILs [23]. The properties of ILs can be tuned by appropriate selection of anions and cations. More than 40 ILs have been investigated in dissolving cellulose. Relatively smalls cations are often effective in dissolving cellulose. Functional groups in the cation can also be crucial for cellulose dissolution. For example, a hydroxyl group in the cation can interact with acetate or chloride anions and compete with cellulose in forming hydrogen bonds [24].

The chloride anion is a stable anion when aiming for cellulose dissolution, whereas large, non-coordinating anions have not been found to be as stable [5]. However, formate and acetate were found to be quite sturdy when combined with an appropriate cation [25]. The dissolution temperature affects the viscosity and the conductivity of ILs. A more intensive stirring effect can be achieved at a higher temperature compared to a lower one. The ILs are exhibiting relatively low viscosities, such as acetate, facilitate dissolution of cellulose at lower temperatures, thus giving a less thermal degradation of cellulose [26].

It has been clarified that ILs have an excellent ability to dissolve cellulose [6,27,28]. The solution may be treated with acid hydrolysis or enzymatic hydrolysis of cellulose, to improve the saccharification rate of dissolving cellulose. However, there is a need to recycle and reuse the ILs. Otherwise, they will have no commercial value.

Table 1.2 Advantages and disadvantages of various pretreatment methods [2]

Pretreatment	Advantages	Disadvantages
Acid (H_2SO_4)	Widely used due to its effectiveness, high sugar recovery efficiency (> 90%) for both xylose and glucose, high hemicellulose solubility, removal of lignin and hemicellulose, cellulose accessibility for enzymatic saccharification	Concentrated-acid process is corrosive and dangerous; specialized non-metallic equipment is needed, formation of inhibitors at low pH, losses of sugar content, neutralization and salt disposal
Alkali (NaOH)	Significant removal of lignin and a part of hemicellulose, decrease in polymerization degree and crystallinity	Low digestibility in softwoods, significant amount of water is needed for washing, long pretreatment resident time, high chemical recovery cost
Ionic liquids (ILs)	Less crystallinity of regenerated cellulose and accessible external and internal surfaces of cellulose, lignin recovery and reuse after removal, disruption of lignin and hemicellulose network	High cost of chemicals, recovery of solubilized cellulose/hemicellulose, toxicity of some ILs, sugar separation from ILs and recycling
Combined methods (microwave-assisted)	Improved enzymatic hydrolysis, efficient removal of lignin and hemicellulose, maximum utilization of lignocellulosic components	High energy demands, specialized equipment is needed, production of toxic waste which can limit further downstream processing, inability to remove hemicelluloses and lignin

1.5 Recycle ILs

The presence of water in the ILs was shown to significantly decrease the solubility of cellulose, presumably through competitive hydrogen-bonding to the cellulose microfibrils, inhibiting solubilization. When water was added to the IL at concentrations greater than ca. 1 wt % (approximately 0.5-mole fraction H_2O), the solvent properties were significantly impaired, and cellulose was no longer soluble [5]. The presence of water significantly hampered the dissolution efficiency of ILs [6].

Dissolved cellulose can be precipitated from the IL solution by adding anti-solvents, such as water, methanol, ethanol, 2-propanol, acetone, dichloromethane, chloroform, acetonitrile, and tetrahydrofuran [5,27]. When anti-solvent is added, the ions of the ILs are extracted into the aqueous phase and are shielded by water molecules, forming hydrodynamic shells. This allows for the direct interaction between the cellulose units in solution [25].

ILs have a very low vapor pressure, thus can be used in high vacuum systems, further reducing environmental problems from volatilization. ILs have good solubility in organic and inorganic minerals. The reaction can occur in homogeneous conditions, thus reducing the volume of equipment. The range of operational temperatures of ILs is between -40 to 300 ºC. They have excellent chemical and thermal stability. ILs could easily be separated from other materials so that they can be recyclable [23]. Swatloski (2002) reported that ILs have high recovery rate and can be re-used [5].

Further research is required to find an economical method that makes use of clean technology for the recovery of ILs at the industrial scale. In this thesis, the recycle and reuse of [Emim]Ac, without any purification of impurities (dissolved materials from wood) was studied.

1.6 Enzymatic saccharification

Due to the robust structure of lignocellulosic biomass, the final purpose of pretreatment is to make the cellulose susceptible to hydrolysis into fermentable sugars [3,4,29,30]. Cost of enzymatic hydrolysis is low compared to acid or alkaline hydrolysis because enzymatic hydrolysis is usually conducted at mild conditions (pH 4.8 and temperature 45–50 ˚C) and does not have a corrosion problem [31].

Cellulose comprises the largest fraction of the sugars in lignocellulose, and glucose is for many microorganisms the preferred carbon source. Enzymatic hydrolysis of cellulose is carried out by cellulase enzymes which are highly specific [32]. However, some pretreatment methods leave the hemicelluloses in the material and efficient hydrolysis of these materials therefore also requires the use of hemicellulases. As hemicelluloses vary between different plant species, the optimal enzyme mixture is most likely to be tailor-made or adjusted to each different kind of material [30].

Cellulases usually consist of enzyme mixtures. At least three groups of cellulases are involved in the hydrolysis process: (1) endoglucanase (EG, endo1,4-D-glucanohydrolase, or EC 3.2.1.4.) which attacks regions of low crystallinity in the cellulose fiber, creating free chain-ends; (2) exoglucanase or cellobiohydrolase (CBH, 1,4-β-D-glucan cellobiohydrolase, or EC 3.2.1.91.) which degrades the molecule further by removing cellobiose units from the free chain-ends; (3) β-glucosidase (EC 3.2.1.21) which hydrolyzes cellobiose to produce glucose. There are also a number of ancillary enzymes that attack hemicelluloses, such as glucuronidase, acetylesterase, xylanase, β-xylosidase, galactomannanase and glucomannanase [3,31].

1.7 Objectives of this study

The IL 1-ethyl-3-methylimidazolium acetate ([Emim]Ac) was used to pretreated lignocellulosic material from softwood which is endemic in Japan (*Cryptomeria japonica*) and water hyacinth (*Eichhornia crassipes*) from the Cirata reservoir, West Java, Indonesia. The pretreatment process is applied to enhance the glucose liberation using enzymatic saccharification. The common pretreatment process using ILs is focused on the dissolution of lignocellulosic materials to produce regenerated cellulose, which requires relatively long steps. But here, we chose the more efficient and straightforward procedure of ILs pretreatment without producing regenerated cellulose.

The thesis is structured as follows:

In Chapter 2, the objective was tested on [Emim]Ac in an efficient pretreatment method, to amorphize cellulose in Japanese softwood material and enhance enzymatic saccharification without collecting regenerated cellulose. This could be a new and more straightforward process for softwoods. The effects of treatment time and temperature were studied. Then, using the proposed method, the recyclability of [Emim]Ac was studied without removing the dissolved materials.

In Chapter 3, the fractions of water hyacinth (stems-leaves, and roots) were characterized and pretreated with [Emim]Ac for enhancing enzymatic saccharification without collecting regenerated cellulose, following the method clarified in Chapter 2. Then, the effects of pretreatment time and enzyme dosage were carefully studied.

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Chapter 2 Recycled ionic liquid 1-ethyl-3-methylimidazolium acetate pretreatment for enhancing enzymatic saccharification of softwood without cellulose regeneration

2.1 Introduction

Wood is a prominent sustainable source of biomass, because of its huge stocks and the fact that it does not directly compete with food production. Materials derived from wood can be used as feedstocks to produce valuable chemicals. In particular, cellulose in the wood can be used to generate biofuels and other bio-based products. The softwood forest in Japan has not been well managed. The amount of softwood plantation in Japan was 3.0 billion m^3 in 2012. However, the self-sufficiency rate of industrial wood is only 31.2% in 2014, and the utilization of wood resources is insufficient [1]. Therefore, it is necessary to develop novel methods to separate and collect wood-derived materials. In this study, we focus on a softwood that is endemic to Japan (*Cryptomeria japonica*). Unfortunately, biotransformation of lignocellulosic biomass is not easy by either microbial or enzymatic routes, thereby limiting its economic conversion into value-added products. The cellulose in wood has a crystalline structure and is covered with lignin and hemicellulose. Therefore, pretreatment is very crucial for the efficient transformation of lignocellulosic biomass.

Several researchers reported the dissolution of lignocellulosic materials in ILs followed by cellulose hydrolysis with acid or enzymes [2,3,4,5,6]. The ILs are liquids at a relatively low temperature (< 100 ºC) consisting of a cation and an anion. They are chemically and thermally stable, non-flammable, non-volatile, and have low vapor pressures. They also have greatly variable chemical and mechanical properties. Hence, ILs have attracted attention as environment-friendly media for chemical reactions and

as solvents in extraction [3,4,7,8]. Swatloski et al. [9] first reported the dissolution of cellulose in ILs, and this was followed by many related studies [10]. Woods, especially softwood, can dissolve in various ILs such as a mixture of 1-butyl-3-methylimidazolium chloride ([Bmim]Cl) and dimethyl sulfoxide (DMSO) [4,5], and then pure cellulose could be separated. Miyafuji et al. [7,11,12] reported that wood components such as cellulose, hemicellulose, and lignin are depolymerized during liquefaction by ILs treatment. The authors concluded that the liquefaction of softwood and hardwood with 1-ethyl-3-methylimidazolium chloride ([Emim]Cl) is not homogeneous from both chemical and morphological viewpoints. The morphological changes were also analyzed using optical and scanning electron microscopy methods [7,11,12].

The ability of ILs to dissolve wood depends on the type of wood, dissolution time, temperature, and IL composition [10]. The dissolution rates of carbohydrates such as cellulose and hemicellulose in wood components are much faster than that of lignin. Consequently, lignin is concentrated in the residue after the dissolution of wood material in an IL, such as [Bmim]Cl [13]. For lignocellulosic materials, 1-ethyl-3-methylimidazolium acetate ([Emim]Ac, **Fig. 2.1**) is among the most promising candidates for industrial applications, due to its non-corrosiveness, non-toxicity, and biodegradability [10]. Mood et al. used five different ILs to treat barley straw and found that [Emim]Ac was the most efficient in cellulose conversion [14]. Both softwood (southern yellow pine) and hardwood (red oak) can be completely dissolved in [Emim]Ac after mild grinding, with red oak dissolving more completely and faster than southern yellow pine [6].

The majority of lignocellulosic pretreatment methods have focused on reducing the lignin content and cellulose crystallinity, without destroying the fermentable sugars

of the lignocellulose [2,15]. It is believed that increasing the accessibility of cellulose is more important than removing lignin for high sugar yields [16,17]. Enzymatic saccharification and acid hydrolysis are used for producing glucose from cellulose. Enzymatic saccharification has some advantages: the hydrolysis can be carried out at lower temperatures, and the glucose liberation is higher than that of acid hydrolysis [2,15]. Factors affecting the enzymatic hydrolysis of cellulose after ILs pretreatment have been studied [2], and cellulose crystallinity is considered a key predictor of the enzymatic saccharification performance [18].

The ILs can be recovered after the regeneration of cellulose with water or water/acetone mixture. The solvent added to the ILs should be evaporated prior to its reuse in the next extraction cycle. Recycling and reusing ILs could help make the process more practical and environment-friendly for industrial applications. [Emim]Ac, which has been demonstrated as an effective ILs with excellent recyclability [15,19], was examined as the solvent in this study.

Herein, [Emim]Ac is tested in an efficient pretreatment method to amorphize cellulose in wood materials for enhancing enzymatic saccharification without collecting regenerated cellulose. This could be a new and simpler process for softwoods. The effects of treatment time and temperature are estimated. Then, using the proposed method, the recyclability of [Emim]Ac was studied without removing the dissolved materials.

2.2 Materials and Methods

2.2.1 Materials

Wood meals (40–80 mesh) from *C. japonica* with a moisture content of around 10% were used for the IL pretreatment. [Emim]Ac with >95% purity was purchased from IoLitec ILs Technologies Inc. Microcrystalline cellulose powder was purchased from Aldrich Chemical Company, Inc., USA. A cellulase mixture (GC220) was provided by Genencore Kyowa Co. Ltd., Japan. Filter paper (Advantec No. 1) was used as a control substrate for comparison.

2.2.2 Separation procedure of [Emim]Ac pretreatment (collecting regenerated cellulose)

The wood meals were previously extracted with ethanol-benzene (1:2, v:v). Afterwards, 1.25 g of oven-dried wood meals were treated with 25 g of [Emim]Ac at 80 °C for 72 h. The mixture was washed with DMSO and acetone, then separated into the residue and filtrate by using a glass filter (1GP16). Excess water was then added to the filtrate to obtain regenerated cellulose in the form of precipitation. The precipitate was separated by centrifugation and washed with water. Meanwhile, the residue was separately washed with water (see the process in **Fig. 2.2(a)**).

2.2.3 Non-separation procedure of [Emim]Ac pretreatment

Wood meals (0.5 g) with 10 g [Emim]Ac were placed in a round flask and heated in an oil bath at 60, 80, and 100 °C for 2, 4, 6, and 8 h, while being mixed with a magnetic stirrer. After the ILs pretreatment, 100 mL of distilled water was added to the flask, and then the mixture was filtered using a 1GP16 glass filter. Residue on the filter was washed with 250 mL of distilled water (see the process in **Fig. 2.2(b)**). After

drying overnight in an oven at 105 °C to remove excess water, the residue was subjected to chemical characterization and enzymatic saccharification.

2.2.4 Recovery and reuse of [Emim]Ac

The [Emim]Ac gathered from softwood pretreatment was reused, without removing the dissolved materials. The subsequent residue and filtrate were obtained using the same procedure as shown in **Fig. 2.2(b)**. As a first step, pristine [Emim]Ac was dried in a vacuum oven at 75 °C for 24 h and used for the initial experimental treatments. Freeze drying was used to remove water from the filtrate containing the recovered [Emim]Ac, and the remainder was further dried in a vacuum oven at 75 °C for 24 h. The recovered [Emim]Ac was directly used for the next cycle of pretreatment without any purification.

2.2.5 Enzymatic saccharification

Untreated and [Emim]Ac-pretreated wood meals (20 mg after oven drying) were suspended in 1.0 mL of 0.05 M acetic acid buffer solution (pH 4.5) with 45 filter paper units (FPU) of GC220 enzyme for each gram of cellulose at 45 °C for 24 h. The enzyme was deactivated by heating at 100 °C for 5 min, and the released glucose was analyzed with an ion chromatograph (ICS 3000, Thermo, USA).

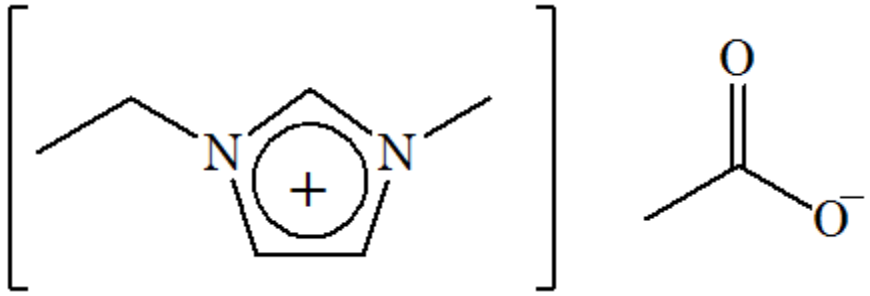

Fig. 2.1 The Chemical structure of 1-Ethyl-3-methylimidazolium acetate ([Emim]Ac)

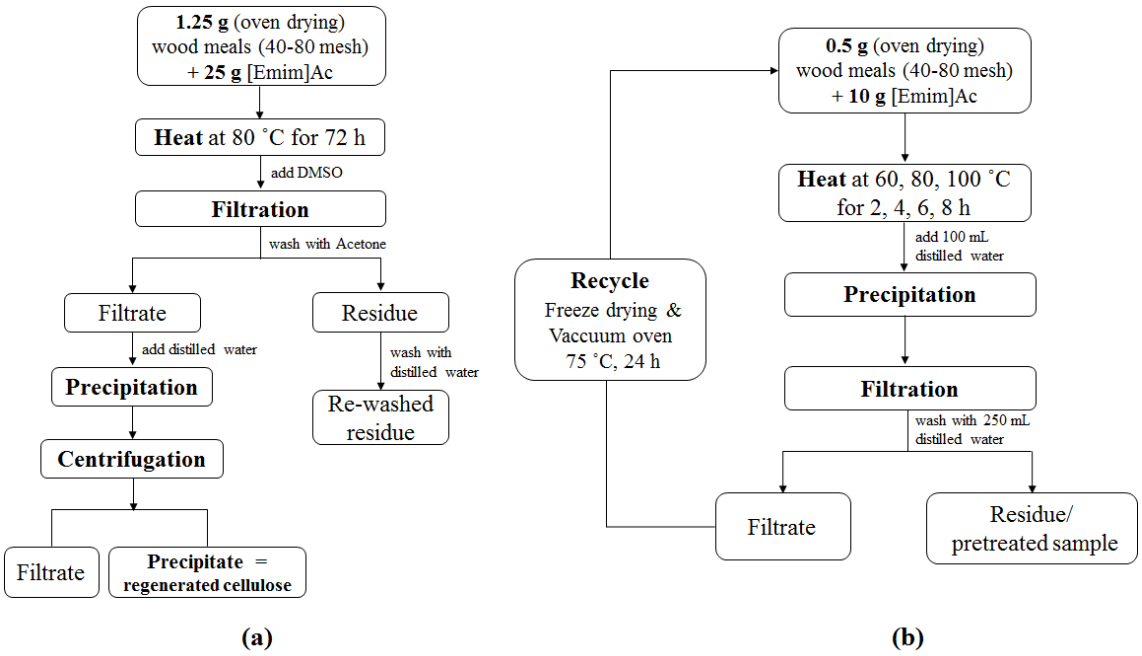

Fig. 2.2 Scheme of [Emim]Ac pretreatment **(a)** separation procedure: collecting regenerated cellulose, **(b)** a propose non-separation procedure: without collecting regenerated cellulose

2.2.6 Analysis of carbohydrates

Glucose liberation values were calculated from the glucose released from enzymatic saccharification relative to glucose contents in the residues. Each residue sample (20 mg after oven drying) was first hydrolyzed with 72% sulfuric acid at room temperature for 2.5 h and then hydrolyzed with 4% sulfuric acid at 121 °C for 1 h. After the hydrolysis, arabinose, galactose, glucose, xylose, and mannose were determined using an ion chromatograph [20]. Specifically, the monosaccharide contents were determined on a Dionex ICS 3000 ion chromatograph (Dionex, Sunnyvale, CA, USA) from a filtrate at 1000-fold dilution. The system consisted of an electrochemical detector (ED), a single pump model (SP-1), and a CarboPac PA 1 column (250 mm × 4 mm i.d.), CarboPac PA 1 guard column (250 mm × 4 mm i.d.), and an autosampler (AS).

2.2.7 Elemental analysis

The adsorption of [Emim]Ac was evaluated by the nitrogen content from the elemental analysis (Perkin-Elmer 2400 CHN Elemental Analyzer from the Research Facility Center for Science and Technology, University of Tsukuba).

2.2.8 Analysis of acid-insoluble lignin (Klason lignin)

Acid-insoluble lignin (Klason lignin) was analyzed by hydrolysis according to the method described elsewhere [21]. A sample was weighed (0.5 g after oven drying) and treated with the two-step hydrolyzation using sulfuric acid as described above. The residue was collected on a glass filter (1GP16), and its weight was measured as acid-insoluble lignin.

2.2.9 Measurement of dissolved lignin in the filtrate

The filtrates were diluted to 500 mL and adjusted to pH 5.0 using acetic acid. The lignin content in the filtrate was determined by UV absorbance at 300 nm, based on the absorption coefficient of 17.2 $L \cdot g^{-1} \cdot cm^{-1}$ obtained from alkali lignin (Aldrich Chemical Company Inc., USA).

2.2.10 X-ray diffraction analysis

Untreated and treated wood meals were sieved to 40–80 mesh size. Their crystallinity was measured by a full-automatic multi-purpose X-ray diffractometer (XRD, D8 Advance/TSM, BrukerTSM, Germany) at 20 °C (voltage 40 kV, 40 mA) with Cu-Kα source (λ = 0.154 nm). The angular range was 10–30° with a step size of 0.03° and a step time of 0.05 s. The XRD data was used to calculate the crystallinity index (C_rI) according to the formula:

$$C_rI = \frac{I_{002} - I_{am}}{I_{002}} \times 100$$

where I_{002} is the maximum intensity of the I_{002} lattice diffraction between 2θ = 21–23°, and I_{am} is the minimum diffraction intensity of the amorphous background between 2θ = 17–19°.

2.3 Results and Discussion

2.3.1 Effect of [Emim]Ac pretreatment on softwood dissolution and cellulose regeneration

The liberated glucose and chemical components of the re-washed residue, the regenerated cellulose, and the filtrate from separation procedure (as explained in **Fig. 2.2(a)**) are shown in **Table 2.1**. The images of the products are shown in **Fig. 2.3**. Pretreatment at 80 °C for a relatively long time (72 h) resulted in a low dissolution ratio of softwood (24.7%). The very low solubility of wood meals in [Emim]Ac contrasts with the high solubility of free cellulose because the presence of lignin lowers the solubility of lignocellulose [2]. The lignin weight of the re-washed residue after 72 h treatment was 25.5%. This indicates that only 26.1% of lignin was dissolved. It was shown that [Emim]Ac pretreatment was not effective for removing lignin, and the same conclusion was reached by Mood et al. [16]. This result differs from that of Lee et al., who used [Emim]Ac to treat maple wood powders to achieve high lignin solubility [15].

Most researchers focus on complete dissolution of lignocellulosic materials in ILs to produce regenerated cellulose, followed by cellulose hydrolysis with acid or enzyme [2,5]. In contrast, in our study, pretreatment at 80 °C for 72 h resulted in low regenerated cellulose (12.6%), as most of the cellulose did not dissolve and remained in the re-washed residue. The yield of the re-washed residue was 75.3% **(Table 2.1)**. These results clarified that the [Emim]Ac pretreatment could not completely dissolve the softwood or effectively produce regenerated cellulose.

(a) (b) (c)

Fig. 2.3 Images of products obtained in the separation procedure of [Emim]Ac pretreatment; (a) filtrate, (b) regenerated cellulose, (c) residue

Table 2.1 Chemical compositions of softwood materials and products obtained in the separation procedure after 1-ethyl-3-methylimidazolium acetate ([Emim]Ac) pretreatment at 80 °C for 72 h

	Yields (%)	Lignin (%)[1)]	Glucan (%)	Mannan (%)	Xylan (%)	Arabinan & galactan (%)	Other (%)	Nitrogen (%)[2)]
Untreated wood	100	34.5	40.9	7.9	5.1	3.2	8.4[3)]	0
Residue	105	26	38.1	5	3.3	1.9	30.8	4.1
Rewashed residue	75.3	25.5	28.3	4	2.5	1.5	13.5	0.9
Regenerated cellulose	12.6	2	5.3	1.2	0.2	0.1	3.8	0.6
Filtrate	12.1	-	0.2	0.1	0.6	0.5	10.7	-

[1)] Klason lignin
[2)] Elemental analysis
[3)] It includes ash content: 0.4%

2.3.2 Enzymatic saccharification of residue and regenerated cellulose

The totals of the liberated residue and regenerated cellulose were more than 100% before re-washing the residue with distilled water. Elemental analysis revealed that [Emim]Ac was adsorbed into the residue. The proportion of adsorbed [Emim]Ac was reduced from 25.1% to 3.9% (based on residue weight) by re-washing using distilled water. The re-washing also increased the degree of glucose liberation of the residue from 51.1% to 90.1%. The digestion of regenerated cellulose (which contained 5.3% [Emim]Ac) was around 100% **(Table 2.2)**. These results indicated that the presence of 25% [Emim]Ac affected the performance of the cellulase, but the cellulase could work effectively when the [Emim]Ac was reduced to <5%. Wang et al. reported that an [Emim]Ac content of <15% was compatible with the cellulase mixture, and a high activity was retained for hydrolyzing Avicel and yellow poplar [22]. Therefore, a small amount of [Emim]Ac does not prohibit the saccharification process. This conclusion could help with the larger-scale applications of the pretreatment process because a specific threshold of residual IL could make the operation more difficult.

The glucose liberation of the re-washed residue (which contained 33.8% lignin) was 90.1%, a value that is higher than that of filter paper (64%) **(Table 2.2)**. The liberation of glucose from the regenerated cellulose was more than 100% (a value which was calculated based on the glucose content obtained from the hydrolysis method using 72% and 4% sulfuric acid), even when the substrate contained 15.8% lignin. Thus, it suggests that the pretreatment using [Emim]Ac can facilitate the enzymatic saccharification of cellulose, either with producing regenerated cellulose or by using the simpler processes.

Table 2.2 Enzymatic saccharification of softwood products obtained in the separation procedure after [Emim]Ac pretreatment at 80 °C for 72 h

	Glucose in 20 mg of initial sample	Lignin content (%)[1)]	[Emim]Ac content (%)[2)]	Liberated glucose (mg)	Glucose liberation (%)[3)]
Filter paper	22.2	-	-	13.8	62.2
Untreated wood	9.1	34.5	-	0.6	6.1
Residue	8.1	26	25.1	4.1	51.1
Re-washed residue	8.3	33.8	3.9	7.5	90.1
Regenerated cellulose	9.3	15.8	5.3	10.4	> 100

1) Klason lignin

2) Calculated from nitrogen content of samples relative to nitrogen content in [Emim]Ac by elemental analysis

3) Calculated from liberated glucose levels generated by enzymatic saccharification relative to glucose content in the pretreated wood meals

2.3.3 Pretreatment without separating regenerated cellulose

Wood meals (0.5 g) were pretreated with 10 g [Emim]Ac using the simpler procedure (i.e., without collecting regenerated cellulose) at 60, 80, and 100 °C for 2, 4, 6, and 8 h, according to the scheme described in **Fig. 2.2(b)**. The results showed a low dissolution of softwood, as 91.7–98.9% of the pretreated wood remained as residues (**Tables 2.3** and **2.4**). The images of untreated and pretreated wood meals are shown in **Fig. 2.4**.

The color of the filtrate became darker with increasing time and temperature of the pretreatment, because of the dissolved wood components in the filtrate. Lignin contents in the filtrate determined with the UV-vis spectrophotometer were 1.9–7.9% of the wood materials. From these results, the lignin contents of the residues at 80 °C after 2, 4, and 8 h were calculated to be 31.4%, 31.0%, and 29.6%, respectively **(Table 2.4)**. Using the same pretreatment time and temperature, lignin contents in the residue were analyzed using the Klason lignin method **(Table 2.3)**, resulting in 31.4%, 30.5%, and 30.9%. Lignin determination by Klason lignin gave results similar to that measured with an indirect method using UV-vis spectrophotometer analysis. Both sets of data demonstrate that lignin contents of the residue remained high.

(a) (b)

Fig. 2.4 Images of wood meals before and after the non-separation procedure of [Emim]Ac pretreatment; (a) untreated, (b) pretreated at 80˚C

Table 2.3 Adsorption of [Emim]Ac relative to the residue obtained in the proposed non-separation procedure

Conditions	Residue weight (%)	[Emim]Ac (%)	Lignin (%)[1)]	Glucan (%)	Mannan (%)	Xylan (%)	Arabinan & galactan (%)	Other (%)
80 ºC, 2 h	96.6 ± 0.5	4.6	31.4	32.4	6.3	4	2.2	15.7
80 ºC, 4 h	95.0 ± 0.2	4.4	30.5	35.5	6.7	4.4	2.3	11.2
80 ºC, 8 h	94.9 ± 0.2	3	30.9	28.3	5.5	3.6	1.9	21.7

[1)] Klason lignin

Table 2.4 Effects of [Emim]Ac pretreatment on the dissolution of softwood components

Pretreatment conditions		Residue weight (%)	[Emim]Ac (%)[1)]	Organic compounds in filtrate		Lignin content in residue (%)[4)]
				Total (%)[2)]	Lignin (%)[3)]	
60 ˚C	2 h	98.8	0.9	2.1	1.9	32.6
	4 h	98.9	1.6	2.7	2.3	32.2
	6 h	98.1	1.3	3.2	3	31.5
	8 h	96.8	1.8	5	3.2	31.3
80 ˚C	2 h	96.6	1.3	4.7	3.1	31.4
	4 h	95	2.2	7.2	3.5	31
	6 h	95	1.6	6.6	4.9	29.6
	8 h	94.9	2.2	7.3	4.9	29.6
100 ˚C	2 h	96.6	1.8	5.2	4.1	30.4
	4 h	94.9	0.9	5.9	5.4	29.1
	6 h	94.5	2.2	7.7	5.7	28.8
	8 h	91.7	2.7	11	7.9	26.6

1) Calculated from nitrogen content of samples relative to nitrogen content in [Emim]Ac by elemental analysis
2) 100 − (residue – [Emim]Ac) (%)
3) UV absorbance at 300 nm
4) 34.5 − lignin content in filtrate (%)

2.3.4 Effects of non-separation pretreatment on glucose liberation and cellulose crystallinity

Separation procedure aimed at producing regenerated cellulose involves relatively long steps. Pretreatment at 80 °C for 72 h, total yield of rewashed residue and regenerated cellulose is 87.9% (**Table 2.1**). The glucose liberated from the rewashed residue reached 90.1% (**Table 2.2**). On the other hand, in a non-separation procedure, at the same temperature (80 °C) and for a relatively short pretreatment period (6–8 h), the glucose released was 40.2–42.6%. Glucose liberation after pretreatment at 100 °C for 8 h was 85.9% (**Table 2.5)**, almost the same as that obtained after a relatively long pretreatment period (80 °C, 72 h). Thus, it can be concluded that the non-separation procedure is also appropriate for glucose liberation.

Two XRD peaks at 15° and 22.5° were clearly observed in the untreated wood samples and microcrystalline cellulose, indicating the presence of crystalline cellulose I [23,24]. The peaks of residues became flattened upon increasing the treatment temperature **(Fig. 2.5)** and time (data not shown). These weak diffraction patterns are attributed mainly to the conversion of crystalline cellulose into amorphous cellulose [23,24]. The C_rI values of microcrystalline cellulose, untreated softwood, and residues were 82.5%, 50.9%, and 37.1–28.4%, respectively.

The lowest glucose liberation in the residues was found to be 16.5% after pretreatment at 60 °C for 4 h (**Table 2.5**). This percentage is still about 3 times that of untreated wood (5.1%). Glucose liberation after pretreatments at 100 °C for 6 and 8 h were 68.6% and 85.1%, respectively. These results are higher than that from filter paper (61.4%). Under moderate pretreatment conditions (80 °C for 6 and 8 h), the respective liberation ratios were 40.3% and 42.6%. The amounts of lignin left in these residues were in the range of 26.6–32.6% **(Table 2.4)**. Hence, the glucose liberation was

successfully increased by the [Emim]Ac pretreatment, even though the residues contained high amounts of lignin.

A good correlation between the decreasing C_rI of cellulose and the increasing enzymatic saccharification efficiency was observed at 80 °C (R^2 = 0.9579) and 100 °C (R^2 = 0.7913). However, the correlation became quite weak at 60 °C (R^2 = 0.2118): pretreatment for 2–8 h reduced C_rI to 23–30%, which did not correlate well with the glucose liberation values. The significant reduction in C_rI confirms that the pretreated samples were highly amorphous, and thus the cellulose surface became much more accessible during enzymatic saccharification [2,15,24]. Ichiura et al. [25] observed that with the partial dissolution of cellulose fibers at 80 °C using a [Bmim]Cl treatment, the resulting C_rI values decreased. Scanning electron microscope (SEM) images further showed that the regenerated cellulose tended to aggregate and form a cellulose film on the surface. Their results may provide an explanation for our findings that suggest that cellulose amorphization, with decreasing C_rI, in the softwood residue was achieved by the pretreatment process with lower solubility.

Table 2.5 Enzymatic saccharification of softwood products obtained in the non-separation procedure after [Emim]Ac pretreatment at various conditions

Pretreatment conditions		Glucose in 20 mg of initial sample	Crystallinity index (%)	Liberated glucose (mg)	Glucose liberation (%)[1)]
60 ˚C	2 h	9.5	38.9	1.7	18.2
	4 h	9.6	39.4	1.6	16.5
	6 h	9.6	36.4	1.9	19.6
	8 h	9.6	35.7	1.7	17.5
80 ˚C	2 h	9.9	35.1	2.8	28.7
	4 h	9.7	33.3	3.5	35.9
	6 h	9.4	30.6	3.8	40.3
	8 h	8.8	30.6	3.8	42.6
100 ˚C	2 h	9.9	29.8	4.5	45.9
	4 h	8.8	28.7	5.3	60.6
	6 h	8.3	28.1	5.7	68.6
	8 h	8.5	28.1	7.3	85.9

[1)] Calculated from liberated glucose levels generated by enzymatic saccharification relative to glucose content of the pretreated wood meals

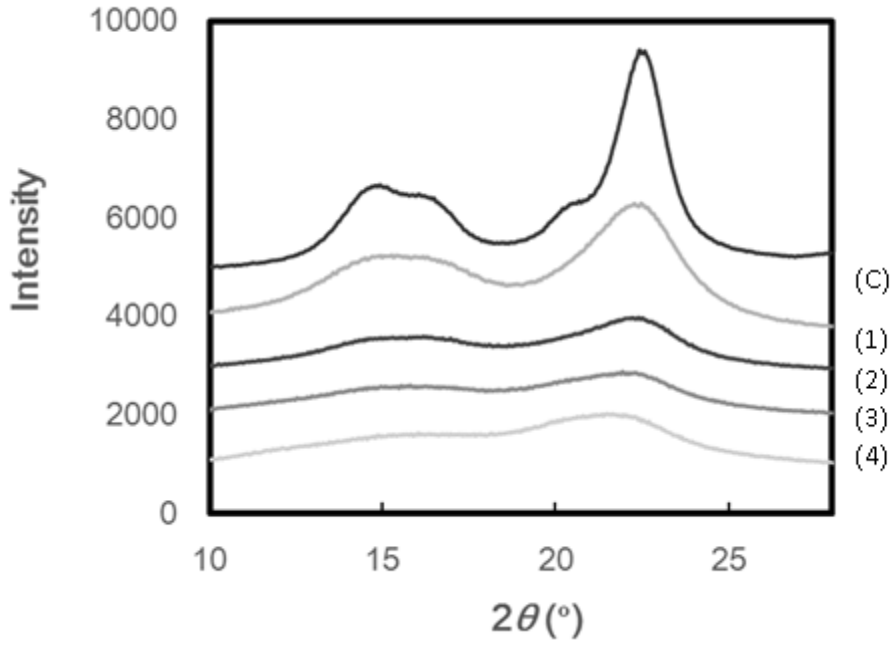

Fig. 2.5 Effects of [Emim]Ac pretreatment on cellulose crystallinity (XRD patterns). Legends: (C) microcrystalline cellulose, (1) untreated wood, (2) pretreated at 60 °C for 6 h, (3) pretreated at 80 °C for 6 h, and (4) pretreated at 100 °C for 6 h

2.3.5 Recovery and reuse of [Emim]Ac

The high-cost of imidazolium cations, which are chemically synthesized from petroleum sources, is one major obstacle to the large-scale industrial application of imidazolium-based ILs for biomass pretreatment [8]. Therefore, the recovery and reuse of ILs are necessary. The presence of water in the ILs is usually detrimental to the dissolution of biomass [8,15,19,25]. For example, the function of recovered [Bmim]Cl was lower than the function of pure [Bmim]Cl if it contained any water. Otherwise, the functionality was similar with pure [Bmim]Cl [25]. In this work, water in the filtrate was removed by a freeze-drying process, and then further dried in a vacuum oven to attain less than 1.0% of moisture content [15,25]. Without further purification, the recovered [Emim]Ac was recycled and reused to pretreat wood meals, resulting in the accumulation of dissolved materials.

Since the influence of moisture on the recycling of IL has already been clarified in many papers, the influence of lignin was examined herein. It is desirable that the lignin content of the organic compounds of filtrate is high. Considering the presence of lignin in the organic compounds of the filtrate, the residue yield (~95%), the medium glucose liberation, crystallinity index (~30%), and low temperature, the recycling experiment was performed at 80 °C for 6 h.

The percentage of lignin in the recovered [Emim]Ac increased with the cycling, reaching 22.4% after the 3rd reuse (which corresponds to an average increase of 5.6% in dissolved lignin per cycle, **Table 6**). The color of the filtrate became darker with the number of reuse of [Emim]Ac, because of the accumulation of dissolved wood components in the filtrate (**Fig. 2.6**). Even though the lignin content in the pretreated wood remained high, and the amount of lignin dissolved in the filtrate increased, the liberation of glucose by enzymatic saccharification was unaffected. Findings showed

that the glucose liberation was equally successful between pristine [Emim]Ac and recovered [Emim]Ac up until the 3rd cycle. This is consistent with the results of Weerachanchai and Lee, who observed the deterioration of reused IL in the 5th–7th batches using thermogravimetric analysis and ^{1}H-nuclear magnetic resonance spectroscopy [19].

Table 2.6 Reuse of [Emim]Ac for softwood pretreatment at 80 ºC for 6 h

Times of reusing [Emim]Ac	Residue weight (%)	Lignin content in filtrate (%)[1)2)]	C_rI (%)	Glucose liberation (%)[3)]
None	95.3	5.5	32.5	48.6
1st	97.8	10.8 (5.3)	32.5	52.6
2nd	97.1	17.3 (6.5)	30.7	49
3rd	96.6	22.4 (5.1)	34	46.2

1) UV absorbance at 300 nm

2) Values in parentheses indicate incremental lignin dissolved for each reuse

3) Calculated from liberated glucose levels generated by enzymatic saccharification relative to glucose content in the pretreated wood meals

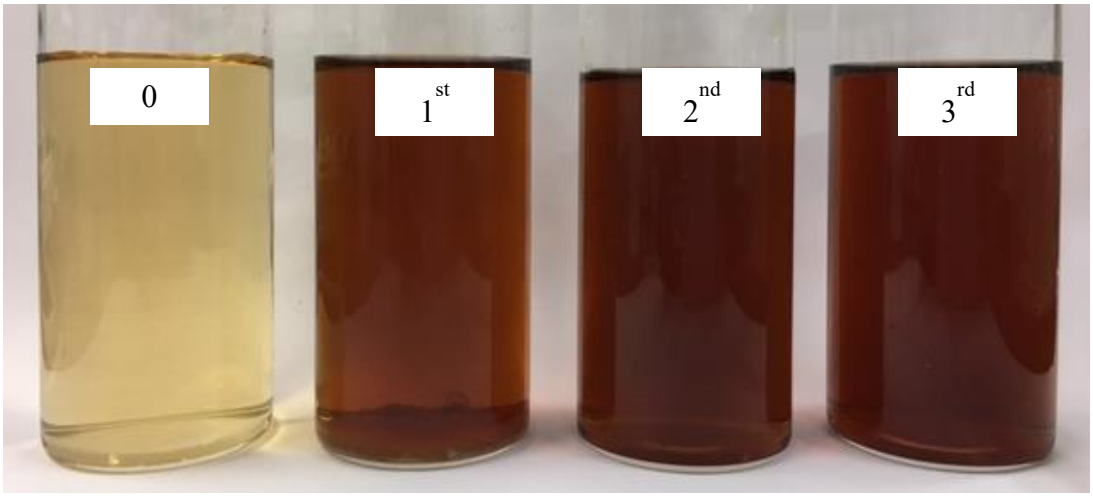

Fig. 2.6 Images of reuse filtrates after [Emim]Ac pretreatment at 80 ºC for 6 h

2.4 Conclusions

It is essential to develop suitable and efficient methods for [Emim]Ac pretreatment of softwood. This study clarified that [Emim]Ac does not have a strong effect on softwood dissolution. Hence, it is not efficient if producing regenerated cellulose and removing lignin are the focuses. Despite the large fraction of residues containing high amounts of lignin, the glucose liberation of pretreated softwood by enzymatic saccharification was significantly increased compared to untreated wood. It was demonstrated that [Emim]Ac substantially reduced the crystallinity of cellulose and amorphized it, thereby increasing the glucose liberation by saccharification. [Emim]Ac was also successfully recycled for at least 3 cycles without performance loss, even though the dissolved lignin accumulated.

2.5 References

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Chapter 4 General Conclusion

In this work, pretreatment of wood meals using a recycled ionic liquid (IL), 1-ethyl-3-methylimidazolium acetate ([Emim]Ac), enhanced glucose liberation by enzymatic saccharification, without dissolution of cellulose and lignin. In contrast, previous studies on IL pretreatment have mostly focused on lignocellulosic dissolution to regenerate cellulose and removing lignin. Softwood (*Cryptomeria japonica*) was pretreated with [Emim]Ac at 60–100 ℃ for 2–8 h without collecting regenerated cellulose. The pretreatment did not have a substantial effect on wood component dissolution (weight of residues: 91.7–98.8%).

The residues contained relatively high amounts of lignin (26.6–32.6%) with low adsorption of [Emim]Ac (0.9-2.7%). Meanwhile, the crystallinity index (C_rI) of cellulose in the woods was significantly reduced by pretreatment, from 50.9% to 28.4–37.1%. In spite of the high lignin contents in the residues, their glucose liberation values by enzymatic saccharification using a cellulase mixture were 3–16-times higher than that of untreated wood. A good correlation was found between the saccharification effectiveness of pretreated samples and the C_rI. Although lignin dissolved in [Emim]Ac continued to accumulate after repeated use of [Emim]Ac; the pretreatment was found to be useful for three consecutive cycles without the need to remove the dissolved materials.

Water hyacinth contains high total sugars and relatively low lignin, which are promising biomass to produce glucose or value-added materials. This study clarified that [Emim]Ac pretreatment without producing regenerated cellulose has an excellent effect on hydrolysis enhancement of all water hyacinth fractions (stems-leaves and roots), despite a significant amount of ash contents in the raw material. Without complete water hyacinth dissolution and treated at a relatively short time (30 and 60

min) at 100˚C, liberated glucose by enzymatic saccharification significantly increased compared with untreated.

Acknowledgements

This PhD thesis becomes a reality with kind support and help of many individuals. First, I am grateful to the Japanese Ministry of Sports and Education for providing a scholarship. I am hugely indebted to my academic supervisor, Associate Professor Akiko Nakagawa-Izumi of her guidance made it possible for me to work and to finish my doctoral course. It was a pleasure to work with her. I am indebted to Professor Hiroshi Ohi for giving his precious and kind advice regarding my research and thesis writing.

I would like to thanks to Professor Toshiharu Enomae and Associate Professor Mikio Kajiyama, for their kinds of advice and great discussions. Special thanks to my co-authors: Dr Masanori Kishino from Hokkaido Research Organization for providing essential materials for this research, Mr Takuya Arakawa and Dr Yin Ying H'ng for helping me to get valuable data.

I am grateful for my laboratory colleagues, for the friendship, the sharing kinds of knowledge and experiences, the many help and the encouragement. Finally, I would like to express my special gratitude to my parents for their never-ending prayers and supports. Special thanks to my beloved husband, Rohimi Ghufron who willing to sacrifice his time to accompany me during PhD period. Special love for my kids, Syauqi-kun, Aufa-kun, Husna-chan and Mahya-chan, thank you for the love and understanding.