

Department of Biological Science and Technology
Tokushima University

A novel pretreatment process
using choline acetate ionic liquid
for effective utilization of lignocellulosic biomass

Ai Asakawa

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PROLUSION

Lignocellulosic biomass is cheap and abundant energy source in nature (Mood et al., 2013), and it is expected as a feedstock in the production of useful substances such as biofuels and bioplastics (Moghaddam et al., 2014; Zhang et al., 2016). However, lignocellulosic biomass has cellulose-hemicelluloses-lignin complex structures and is recalcitrant to breakdown and offers limited accessibility to enzymes or microorganisms (Prez-Pimienta et al., 2013). Therefore, the useful substances production from lignocellulosic biomass through enzymatic saccharification and fermentation requires a pretreatment step before saccharification and fermentation (Mai et al., 2014). The aim of the pretreatment is to increase the accessibility of cellulase enzymes to cellulose during enzymatic saccharification, e.g., by removing lignin and/or hemicelluloses (Uju et al., 2012). Numerous pretreatments have been reported; however, most of all are not put to practical use due to problems such as high energy requirements or high cost of the solvents used for processing and recycling (Ortiz and Oliveira Jr, 2014).

In this study, we propose a novel ionic liquid (IL) pretreatment using choline acetate ([Cho][OAc]). ILs are organic salts and exist in liquid form below 100°C (Nasipour et al., 2014; Cheng et al., 2014), and they have characteristics, such as high polarity, high thermal stability, and non-volatility, and can dissolve polar and non-polar organic, inorganic, and polymeric compounds including cellulose under mild conditions (Weerachanchai et al., 2012; Vancov et al., 2012; Ninomiya et al. 2013).

In CHAPTER 1, to evaluate the effects of the pretreatment using [Cho][OAc], componential analysis and monitoring the enzymatic saccharification of the [Cho][OAc]-pretreated biomass, and calculation of the energy profit ratio (EPR) with the pretreatment were carried out. And then, these results were compared with those of various pretreatment methods, such as mechanical comminution, microwave irradiation, and alkaline treatment. Sugarcane bagasse was selected as the lignocellulosic biomass, which is an agricultural residue that is unutilized except for producing steam and electricity in sugarcane processing plants (Martín et al., 2002); thus, a more beneficial use of this substrate is

desirable.

In CHAPTER 2, to reduce the use of [Cho][OAc], the pretreatment combined use of [Cho][OAc] and organic solvent was attempted. First, the sugarcane bagasse pretreated with the mixtures of [Cho][OAc] and several polar organic solvents were enzymatically saccharified, and their hydrolizabilities were investigated. Secondly, to demonstrate that the influence of the [Cho][OAc]/cosolvents pretreatment on the chemical composition of bagasse and cellulose, the componential analysis of [Cho][OAc]/cosolvents-pretreated bagasse and the measurement of the recovery and cellulose crystallinity index (CrI) of pretreated microcrystalline cellulose were conducted. In addition, ethanol fermentation was performed to investigate the fermentability of the [Cho][OAc]/cosolvent-pretreated bagasse. Aprotic polar solvents such as N, N-dimethylacetamide (DMA), N, N-dimethylformamide (DMF), dimethyl sulfoxide (DMSO), and N-methyl-2-pyrrolidone (NMP), and protic polar solvents such as ethylene glycol (EG) and glycerin (G) were used as organic solvents.

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CHAPTER 1. General introduction

1.1. Production of useful materials from lignocellulosic biomass

The increase in demand for energy, a depletion and unstable supply of petroleum, and global warming's threat have generated a great deal of interest in pursuing alternative and renewable energy sources (Zhu et al., 2012).

Biomass is defined as organic matter available on a renewable basis, and includes forest residue, agricultural crops and wastes, wood and wood wastes, animal wastes, aquatic plants, and municipal and industrial wastes (Vancov et al., 2012). Biofuel produced from the biomass could help reduce the dependence on oil, and have the potential to cut CO₂ emission because the plants are made from use CO₂ as they grow (Vancov et al., 2012; Naik et al., 2010). Biofuel includes bio-alcohols (ethanol, butanol etc.), biodiesel, bio-oils, and syngas derivatives. Particularly, lignocellulosic biomasses, such as agricultural and woods residues, and biofuel crop, have received much attention as a feedstock for biofuel production, because they are cheap and abundant energy source in nature (Mood et al., 2013). Lignocellulosic biomass consists of three main components: 35-50% cellulose, 20-35% hemicelluloses, and 15-20% lignin (Mood et al., 2013).

Cellulose (C₆H₁₀O₅)_x is a linear chain of D-glucose linked by β-1,4 glycosidic bonds to each other, and the individual cellulose chains are associated together by strong hydrogen bonds and Vander Waals forces, resulting in microfibrils with high tensile strength (Mood et al., 2013; Wang et al., 2015; Zheng et al., 2014). Cellulose consists of two regions: amorphous (low crystallinity) and crystalline (high crystallinity) regions (Zheng et al., 2014). In the X-ray diffraction diagram, the natural cellulose and the mercerized cellulose show type I and type II, respectively. Cellulose type III is obtained by removal ammonia after swelling with the ammonia. Besides, heating cellulose type I or III in glycerin makes type IV. Like these, cellulose has a polycrystalline structure (Jodai and Samejima, 1993)

On the other hand, hemicelluloses (C₅H₈O₄)_m are heterogeneous branched biopolymers containing

pentoses (β -D-xylose, α -L-arabinose), hexoses (β -D-mannose, β -D-glucose, β -D-galactose) and/or uronic acids (α -D-glucuronic, α -D-4-O-methyl-galacturonic and α -D-galacturonic acid). They are relatively easy to hydrolyze because of their amorphous and branched structure (with short lateral chain) as well as their lower molecular weight (Mood et al., 2013). Short and branched chains of hemicelluloses help build a network with cellulose microfibrils and interact with lignin, rendering the cellulose-hemicelluloses-lignin matrix extremely rigid (Zheng et al., 2014). In other words, hemicelluloses cover cellulose fibrils limiting their availability for enzymatic hydrolysis; therefore, removal of large amounts of hemicelluloses is necessary in order to increase the digestibility of cellulose (Mood et al., 2013).

Lignin is a heterogeneous, aromatic and amorphous copolymer binding hemicelluloses and cellulose by ester linkages and hydrogen bonds, respectively. Lignin has three monolignol monomers, methoxylated to various degrees: *p*-coumaryl, coniferyl and sinapyl alcohols, which exist in lignin as three basic substructure units: *p*-hydroxyphenyl, guaiacyl and syringyl units, respectively (Moghaddam et al., 2014). The measurement of degree of polymerization of the natural lignin is difficult; therefore, the accurate structure of lignin is not yet become clear (Moghaddam et al., 2014). Lignin is water insoluble, and begins to dissolve in water at high temperature (e.g. 180°C). Lignin plays the role of cement for the cross-linking between cellulose and hemicelluloses to form a rigid three-dimensional structure of the cell wall. These properties of lignin make it the most recalcitrant component of the plant cell wall, and the lignin is a major barrier to utilization of lignocellulosic biomass in bioconversion processes (Zheng et al., 2014).

As expressed up until now, lignocellulosic biomass has cellulose-hemicelluloses-lignin complex structures and is recalcitrant to breakdown and offers limited accessibility to enzymes or microorganisms (Prez-Pimienta et al., 2013). For this reason, production of biofuels or chemicals from lignocellulosic biomass through enzymatic saccharification requires pretreatment step before saccharification and fermentation (Mai et al., 2014). The aim of the pretreatment is to increase the

accessibility of cellulase enzymes to cellulose during enzymatic saccharification, i.e., by increasing the surface area of cellulose, decreasing the cellulose crystallinity, and removing lignin and/or hemicelluloses (Uju et al., 2012; Yoon et al., 2012; Li et al., 2010b).

1.2. Pretreatments of lignocellulosic biomass for the production of useful materials

An effective pretreatment must fulfill the following requirements: (1) liberating fermentable sugars (e.g. glucose, xylose), (2) avoidance significant degradation or loss of sugars, (3) avoidance the formation of byproducts which inhibit the subsequent saccharification and fermentation (Perez-Pimienta et al., 2013). Inhibitory byproducts found in hydrolysates include acetic acid, formic acid, levulinic acid, furfural, 5-hydroxymethyl-2-furaldehyde (HMF), vanillin, syringaldehyde, and coniferyl aldehyde (Zhang et al., 2015)

Numerous pretreatments, such as biological (Sasaki et al., 2011), physical (Zheng et al., 2014), chemical (Masarin et al., 2013), and physicochemical (Asada et al., 2011) pretreatments, are reported; however, they are all affected by problems such as long residence time, high energy requirements, or high cost of the solvents used for processing and recycling (Ortiz and Oliveira Jr, 2014). Thus, the development of an innovative pretreatment method is necessary to improve the commercial viability of this process.

1.3. Ionic liquid pretreatment using choline acetate

Ionic liquid (IL) pretreatment, which is one of the chemical pretreatment, has received much attention since some sorts of ILs were discovered to be able to dissolve cellulose (Swatloski et al., 2002). ILs are organic salts usually composed of organic cations and inorganic anions, and exist in liquid form below 100°C (Nasipour et al., 2014; Cheng et al., 2014). ILs have characteristics, such as high thermal stability, high ionic conductivity, miscibility, water stability, density, viscosity, polarity, non-volatility. Besides, ILs can dissolve polar and non-polar organic, inorganic, and polymeric

compounds, including hemicelluloses, lignin, and cellulose, under mild conditions (Vancov et al., 2012; Ninomiya et al. 2013b). Cellulose is very difficult to dissolve because of the extensive network of inter- and intra-molecular hydrogen bonds and van der Waals interactions between cellulose fibrils (Vancov et al., 2012) In, general, the anions in IL function as hydrogen bond acceptors, which interact with the hydroxyl groups in cellulose to make the crystalline structure of cellulose vulnerable, while the cations interact with lignin via hydrogen bonding and π - π interactions (Ninomiya et al., 2013c; Wu et al., 2011). Therefore, ILs can partially remove hemicelluloses and lignin and reduce the crystallinity of cellulose, which greatly improves the accessibility of cellulase (Uju et al., 2012; Li et al., 2010a; Zheng et al., 2014; Dadi et al., 2006). It was also suggested that ILs could be recycled and reused (Uju et al., 2013; Ohno and Fukuya, 2009).

Imidazolium ILs have been studied widely in particular, thereby demonstrating their powerful enhancement effects on enzymatic saccharification (Silva et al., 2011). However, imidazolium ILs are derived from petroleum; thus, they have disadvantages such as low biodegradability, cytotoxicity, and high cost (Ninomiya et al., 2013c; Datta et al., 2010).

To overcome these disadvantages of imidazolium ILs, choline acetate ([Cho][OAc], Fig.1) was focused on in the present study. [Cho][OAc] consists of cholinium cations and acetate anions, and the former are derived from choline chloride, which is part of the vitamin-B complex, the latter are derived from intercellular metabolites (Ninomiya et al., 2013a). That is, [Cho][OAc] is a completely bioderived IL, and is easy to synthesize, less expensive, more biodegradable, and more biocompatible than imidazolium ILs (Ninomiya et al., 2015; Boething et al., 2007). Ninomiya et al. (2013c) reported that [Cho][OAc] pretreatment facilitated almost 100% conversion from cellulose into glucose using kenaf powder after 48-h enzymatic saccharification, which is comparable with the results obtained with 1-ethyl-3-methylimidazolium acetate. However, few studies have assessed the use of [Cho][OAc] as a pretreatment for lignocellulose, which remains to be fully elucidated.

In the present study, the effect of IL pretreatment using [Cho][OAc] was compared with various

pretreatments, such as mechanical comminution, microwave irradiation, and alkaline treatment. To evaluate these pretreatments effects, component analysis of the pretreated lignocellulose, monitoring the subsequent enzymatic saccharification, and calculation of the energy profit ratio (EPR) with pretreatment were carried out. For calculation of the EPR, the theoretical ethanol yield was estimated based on the glucose yield obtained from enzymatic saccharification. Sugarcane bagasse was selected as the lignocellulosic material, which is an agricultural residue that is unutilized except for producing steam and electricity in sugarcane processing plants (Martín et al., 2002); thus, a more beneficial use of this substrate is desirable.

1.4. Pretreatment combined [Cho][OAc] IL with cosolvent

The high effect of [Cho][OAc] IL pretreatment is becoming clear as described above. Although [Cho][OAc] is relatively inexpensive among ILs, it is generally expensive, therefore a further cost cut is demanded for practical use. Furthermore, ILs/biomass solution is high viscosity, which makes it difficult to handle and to increase biomass loading (Mood et al., 2013; Fu and Mazza, 2011; Wang et al., 2015). Particularly, [Cho][OAc] is more difficult to mix with biomass because of the solid condition of [Cho][OAc] at room temperature.

In order to overcome these problems, the pretreatment combined use of [Cho][OAc] with polar organic solvent was proposed in this work. It has been revealed that the pretreatment using organic solvent have the advantages: (1) cellulose was recovered as the solids with only minor degradation, (2) lignin and hemicelluloses dissolved into organic solvent, which increased surface area of cellulose, (3) organic solvent could be easily recovered by distillation and recycled (Zhang et al., 2015).

To evaluate the efficiency of the pretreatment combined [Cho][OAc] with cosolvent, the following experiments were conducted. Sugarcane bagasse pretreated with the mixtures of [Cho][OAc] and several organic solvents were enzymatically saccharified, and their hydrolyzabilities were investigated. And then, to reveal the effect of the pretreatments on the chemical composition of bagasse and

cellulose, the componential analysis of [Cho][OAc]/cosolvents-pretreated bagasse and the determinations of the recovery and crystallinity index (CrI) of microcrystalline cellulose after the [Cho][OAc]/cosolvent treatment were performed. Additionally, simultaneous saccharification and fermentation (SSF) was performed to investigate the fermentability of the [Cho][OAc]/cosolvent-pretreated bagasse. Aprotic polar solvents such as dimethyl sulfoxide (DMSO), N, N-dimethylformamide (DMF), N,N-dimethylacetamide (DMA), and N-methyl-2-pyrrolidone (NMP), and protic polar solvents such as ethylene glycol (EG) and glycerol (G) were used as organic cosolvents.

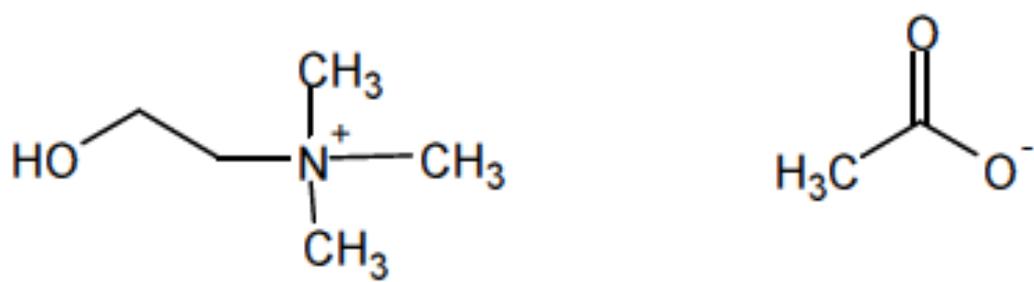


Fig.1 Choline acetate

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CHAPTER 2. Comparison of choline acetate ionic liquid pretreatment with various pretreatments for enhancing the enzymatic saccharification of sugarcane bagasse

2.1. Introduction

Lignocellulose the most abundant organic material on earth, and is generally unutilized, therefore it is expected as a feedstock in the production of useful materials such as biofuels and bioplastics (Palonen et al., 2004; Qiu et al., 2012). Initially, cellulose needs to be converted into glucose during the production of these materials. However, this is difficult because the cellulose in the lignocellulose has a crystalline structure, which is covered by the robust and complex structure of lignin and hemicellulose (Uju et al., 2013). Numerous pretreatments have been used to increase the accessibility of cellulose for cellulase enzymes during enzymatic saccharification, i.e., by increasing the surface area of cellulose, decreasing the cellulose crystallinity, and removing lignin and/or hemicellulose (Yoon et al., 2012; Li et al., 2010b). Biological (Sasaki et al., 2011), physical (Zheng et al., 2014), chemical (Masarin et al., 2013), and physicochemical (Asada et al., 2011) pretreatments have been reported; however, they are all affected by problems such as long residence time, high energy requirements, or high cost of the solvents used for processing and recycling (Ortiz and Oliveira Jr, 2014). Thus, the development of an innovative pretreatment method is necessary to improve the commercial viability of this process.

Studies of pretreatment using ionic liquids (IL) have continued to rise since Swatloski et al. (2002) have reported that imidazolium-ILs can dissolve cellulose. ILs are capable of dissolving hemicellulose, lignin, and cellulose in biomass, thereby allowing the removal of hemicellulose and lignin and reduction the crystallinity of cellulose (Uju et al., 2012; Li et al., 2010a). Thus, the accessibility of cellulase is greatly improved, which increases saccharification rate and the sugar yield (Zheng et al., 2014; Dadi et al., 2006). In addition, it is been suggested that ILs could be recycled and reused because they have low melting points, and they are also nonvolatile and thermally stable (Uju et al.,

2013; Ohno and Fukuya, 2009).

In particular, imidazolium ILs have been widely studied, thereby demonstrating their powerful effects on enhanced enzymatic saccharification (Silva et al., 2011). However, imidazolium ILs are derived from petroleum; thus, they have disadvantages such as low biodegradability, cytotoxicity, and high cost (Ninomiya et al., 2013c; Datta et al., 2010). In the present study, choline acetate ([Cho][OAc], Fig. 1) was focused on as an alternative to imidazolium ILs. [Cho][OAc] consists of cholinium cations and acetate anions, and the former are derived from choline chloride, which is part of the vitamin-B complex, the latter are derived from intercellular metabolites (Ninomiya et al., 2013a). That is, [Cho][OAc] is a completely bioderived IL, which is more biodegradable, biocompatible, and less expensive than imidazolium ILs (Ninomiya et al., 2015; Boething et al., 2007). Furthermore, Ninomiya et al. (2013c) reported that [Cho][OAc] pretreatment facilitated almost 100% cellulose conversion using kenaf powder after 48-h enzymatic saccharification, which is comparable with the results obtained with 1-ethyl-3-methylimidazolium acetate. However, few studies have assessed the use of [Cho][OAc] as a pretreatment for lignocellulose, which remains to be fully elucidated.

In the present study, the effects of IL pretreatment using [Cho][OAc] was compared with various methods, such as mechanical comminution, microwave irradiation, and alkaline treatment. To determine the most effective pretreatment method in terms of enzymatic saccharification using the pretreated lignocellulosic material, the subsequent enzymatic saccharification was monitored and the energy profit ratio (EPR) with each different pretreatment were calculated. To calculate EPR, the theoretical ethanol yield was estimated based on the glucose yield obtained from enzymatic saccharification. Sugarcane bagasse was selected as the lignocellulosic material, which is an agricultural residue that is unutilized except for producing steam and electricity in sugarcane processing plants (Martín et al., 2002); thus, a more beneficial use of this substrate is desirable.

2.2. Materials and methods

2.2.1. Raw material

The sugarcane bagasse was kindly provided by Kyuyo Sugar Industry (Okinawa, Japan), and it was used as the lignocellulosic material. The raw bagasse was ground for 1 min and passed through a 500- μm sieve to obtain particles with a uniform size, which were then used in all the pretreatment tests, except the comminution pretreatment.

2.2.2. IL pretreatment using [Cho][OAc]

[Cho][OAc] with a melting point of 85°C was purchased from Sigma-Aldrich Japan Co. LLC. (Osaka, Japan).

1.5 g of the untreated bagasse was placed in a 300-ml eggplant flask with 1.5, 3.0 and 4.5 g of [Cho][OAc] and incubated in an oil bath at 90°C, 110°C, and 130°C for 60, 180, and 360 min, respectively. Mixing was applied at 30 min after the beginning of heating and then every 60 min. Following incubation, 135 ml of distilled water was added, and the residue was separated from the mixture by filtration, which was then washed thoroughly with an equal volume of distilled water four times. The residue on the filter paper was collected and stored at 4°C for subsequent enzymatic saccharification. Before analyzing the composition and obtaining gravimetric measurements, a part of the residue on the filter paper was dried overnight at 105°C \pm 3°C.

2.2.3. Specific heat capacity determination by differential scanning calorimetry

To calculate the energy requirement for pretreatment using [Cho][OAc], the specific heat capacity of [Cho][OAc] was determined by differential scanning calorimetry (DSC) (DSC6220; Hitachi High-Tech Science Corporation, Tokyo, Japan). The measurements were performed three times under identical conditions. The first measurement was performed with two empty crucibles, the second with the test sample ([Cho][OAc]) and the third with the reference substance (Al_2O_3). The resulting heat

flux curves are the basis for determining the value of the specific heat capacity C_p , which was calculated from the following relationship (Przeliorz et al., 2014):

$$C_{P_s}(T) = \frac{HF_{\text{sample}} - HF_{\text{blank}}}{HF_{\text{ref.}} - HF_{\text{blank}}} \cdot \frac{m_{\text{ref.}}}{m_{\text{sample}}} \cdot C_{P_{\text{ref.}}}(T)$$

where C_{P_s} is the specific heat capacity of the test sample, $J/g \cdot K$; HF is the heat flux respectively for test sample (sample), empty crucibles (blank) and reference substance (ref.), μV ; m is the mass of the sample and the reference substance, g; $C_{P_{\text{ref.}}}$ is the heat capacity of the reference substance, $J/g \cdot K$.

The measurements were performed using 3.5 mg of the samples sealed in stainless steel crucibles and in the temperature range of 25°C-130°C at a heating rate of 10°C/min under N_2 . As the heat capacity of the reference substance (Al_2O_3), the specific heat capacity of $\alpha-Al_2O_3$ (JIS K 7123) was used.

2.2.4. Mechanical comminution

A SAMPLE MILL (a rod mill with two pots (250 ml) and a power consumption of 750 W; TI-300, CMT Co., Ltd., Fukushima, Japan) was used for the mechanical comminution pretreatment. Approximately 40 g (dry weight) of raw bagasse was placed in each pot, which was then ground for 10, 20, 30, or 60 min. The pretreated samples were stored at room temperature for enzymatic saccharification and dried overnight at $105 \pm 3^\circ C$ to analyze their compositions.

2.2.5. Microwave irradiation

Microwave irradiation pretreatment was performed at 2.45 GHz using an Initiator+8 (maximum temperature of 200°C; Biotage Japan Co., Ltd, Tokyo, Japan) with a 30-ml reaction vial. First, 0.9 g (dry weight) of untreated bagasse was suspended in 16.2 ml of distilled water and irradiated at 150°C, 170°C, or 200°C for 1, 3, 5, or 10 min with stirring. Furthermore, 81 ml of distilled water was added, and the residue was separated from the mixture by filtration, before washing several times with 81 ml

of distilled water. The residue on the filter paper was collected and stored at 4°C for subsequent enzymatic saccharification. A part of the residue on the filter paper was dried overnight at 105°C ± 3°C to analyze the composition and to obtain gravimetric measurements.

2.2.6. Alkaline pretreatment

In this pretreatment, 1.5 g (dry weight) of untreated bagasse was suspended in 30 ml of sodium hydroxide (NaOH) at concentrations of 0.25%, 0.5%, 1.0%, or 3.0% (w/v), and the mixture was heated in an autoclave at 121°C for 5, 15, 30, or 60 min. After cooling to room temperature, the mixture was neutralized with acetic acid and stirred by a magnetic stirrer for 1 h. The residue was separated by centrifugation and washed with distilled water several times. The residue was collected and stored at 4°C for subsequent enzymatic saccharification. A part of the residue on the filter paper was dried overnight at 105°C ± 3°C before analyzing the composition and obtaining gravimetric measurements.

2.2.7. Component analysis of untreated and pretreated bagasse

The acid-soluble lignin (ASL), acid-insoluble lignin (AIL), cellulose, and hemicellulose contents of untreated and pretreated bagasse were determined as shown in Fig.2.

First, 3 ml of 72% (w/w) H₂SO₄ was added to 0.2 g of the sample, and the solution was left at room temperature for 4 h. Furthermore, the mixture was diluted to 4% (w/w) H₂SO₄ and autoclaved at 121°C for 60 min. The ASL content was determined from the UV absorbance of this hydrolyzed solution at 205 nm using an absorption coefficient of 110 L g⁻¹ cm⁻¹. The cellulose content was determined based on the monomer content (glucose) in the hydrolyzate. The glucose content was determined using HPLC equipment with a refractive index detector (RID-10A, Shimadzu Co., Ltd., Kyoto, Japan) with an Aminex column (HPX-87H; Bio-Rad, Richmond, CA) at 65°C, with 5.0 mM H₂SO₄ as the mobile phase at 0.6 ml/min. The hemicellulose content was determined by subtracting

the cellulose content from the holocellulose content. The holocellulose content was determined using the phenol–sulfuric acid method. The insoluble residue in the hydrolysate, i.e., AIL (high molecular weight lignin), was washed, dried to constant weight at 105°C ± 3°C, and weighed. All the analytical determinations were performed in triplicate, and the means were calculated.

2.2.8. Enzymatic saccharification of pretreated bagasse

Untreated and pretreated bagasse were hydrolyzed enzymatically using Meicelase (derived from *Trichoderma viride*; Meiji Seika Pharma Co., Ltd, Tokyo, Japan). Enzymatic saccharification was performed with 100 mM sodium acetate buffer (pH 5.0) at 50°C on a rotary shaker at 140 rpm for 72 h. The substrate concentration and enzyme loading were 1% (w/v) and 0.1% (w/v), respectively. The glucose concentration was determined at specific time intervals using an enzymatic determination glucose assay kit (Autokit Glucose, Wako Pure Chemical Industries Ltd., Osaka, Japan). The saccharification ratio and glucose yield were calculated using the following equations:

$$\text{Saccharification ratio (\%)} = \frac{\text{Glucose produced (g/L)} \times 0.9}{\text{Cellulose in pretreated sample (g/L)}} \times 100$$

$$\text{Glucose yield (g/g – bagasse)} = \frac{\text{Glucose produced (g)}}{\text{Raw bagasse before pretreatment(g)}}$$

All the enzymatic saccharification experiments were performed in triplicate, and the means were calculated.

2.2.9. EPR with various pretreatments

Assuming that all the glucose obtained by enzymatic saccharification was converted into ethanol, the energy production from 1 g of raw bagasse was calculated according to the following equations:

$$\text{Theoretical ethanol yield (g/g – bagasse)} = \text{Glucose yield (g/g – bagasse)} \times 0.511$$

$$\text{Energy production (kJ/g – bagasse)} = \text{Theoretical ethanol yield (g/g – bagasse)} \times 29.75 \text{ (kJ/g)}$$

where 0.511 is the theoretical ethanol conversion ratio and 29.75 kJ/g is the combustion heat of ethanol.

The energy requirements of various pretreatments were also calculated. For mechanical comminution, the energy required to treat 1 g of the raw bagasse was calculated according to the following equation:

$$\text{Energy requirement in the comminution pretreatment (J/g – bagasse)}$$

$$= \frac{\text{Power consumption (W)} \times \text{Treatment time (s)}}{\text{Sample (g)}}$$

For the pretreatments using IL, microwave irradiation, and alkaline treatment, the energy required to treat 1 g of raw bagasse was calculated by summing the energy requirements for bagasse and solvents, i.e., IL and distilled water. The energy required to heat 1 g of bagasse was calculated using the specific heat of wood (Sobue, 2006; Kollmann et al., 1968; McMillin, 1970) according to the following equations:

$$\text{Specific heat of wood (J/g} \cdot \text{K)} = \frac{\text{Moisture content (\%)} + 32.4}{100 + \text{Moisture content (\%)}} \times 4.186$$

$$\text{Energy requirement for heating bagasse (J/g – bagasse)}$$

$$= \text{Specific heat of wood (J/g} \cdot \text{K)} \times \{ \text{treatment temperature (K)} \\ - \text{room temperature (K)} \}$$

where the moisture content of bagasse was 3.6%, which was determined by measuring the moisture content. The energy required to heat 1 g of distilled water was calculated using the specific enthalpy of water according to the following equation:

$$\begin{aligned} &\text{Energy requirement for heating distilled water (J/g – distilled water)} \\ &= \text{Specific enthalpy of water at a treatment temperature (J/g)} \\ &\quad - \text{Specific enthalpy of water at a room temperature (J/g)} \end{aligned}$$

The energy required to heat 1 g of [Cho][OAc] was determined based on the specific heat capacity measurement obtained by DSC.

The EPR was calculated using these values as follows:

$$\text{EPR} = \frac{\text{Energy production (kJ/g – bagasse)}}{\text{Energy requirement for pretreatment (kJ/g – bagasse)}}$$

2.3. Results and discussion

2.3.1. Effect of [Cho][OAc] IL pretreatment on the composition of bagasse compared with other pretreatments

First, to optimize the amount of [Cho][OAc] for pretreatment, the pretreatment was performed using [Cho][OAc] (g)/bagasse (g) at ratios of 1, 2, and 3 at the condition of 110°C for 180 min. In the pretreatment of bamboo powder using [Cho][OAc], that the cellulose saccharification ratio was increased with the increase IL/biomass ratio when the IL/biomass ratio was <3, and was reduced when the IL/biomass ratio was >3 (Ninomiya et al., 2013b). However, sugarcane bagasse was easy to saccharify compared to bamboo from our enzymatic saccharification experiment using comminuted samples, therefore, IL/biomass ratio of 1, 2 and 3 were investigated in the present study. After 72 h

enzymatic saccharification, the saccharification ratios of IL/biomass ratio of 1, 2 and 3 were 45.2%, 63.9% and 77.2%, respectively. The maximum glucose yield was obtained using a [Cho][OAc]/bagasse ratio of 3, which was employed in the subsequent experiments.

To investigate the effects of the IL pretreatment with [Cho][OAc] and the other pretreatments, i.e., comminution, microwave irradiation, and alkaline treatment, on the composition of bagasse, the compositions of the untreated and pretreated bagasse were analyzed, and the results are shown in Fig.3 (for the pretreatments other than [Cho][OAc] pretreatment, the results obtained in the conditions with the maximum glucose yield after enzymatic saccharification are only shown). The composition of the untreated bagasse was 36.8% cellulose, 30.0% hemicellulose, 26.2% AIL, and 0.4% ASL.

With the [Cho][OAc] pretreatment, the gravimetric recovery of bagasse decreased with the increasing severity of the treatment condition, i.e., the lowest recovery was 78.8% at 110°C for 360 min and 130°C for 180 min. In these conditions, the AIL and hemicellulose contents reduced to 15.5–17.1% and 20.3–21.9%, respectively. However, the cellulose content decreased only slightly to 34.2% (110°C for 360 min) and 32.7% (130°C for 180 min), respectively, and most of it remained. These results show that the [Cho][OAc] pretreatment was effective in removing hemicellulose and lignin, where the effect increased with the severity of the conditions. It is well known that the removal of lignin and/or hemicellulose enhances the subsequent enzymatic saccharification by improving the accessibility of cellulase enzyme (Li et al., 2010b).

The reduction in AIL using the [Cho][OAc] pretreatment was lower than that with the alkaline treatment (8.4% at 0.5% of NaOH and 121°C for 15 min), which facilitated the delignification of lignocellulose (McIntosh and Vancov, 2010, Sun and Cheng, 2002). Using the [Cho][OAc] pretreatment, the reduction in hemicellulose was less than that with the microwave irradiation pretreatment (4.5% at 200°C for 10 min). It has been reported that microwave irradiation has thermal and non-thermal effects, which caused fragmentation and swelling, leading to degradation of lignin and hemicellulose (Peng et al., 2013). The AIL and hemicellulose contents changed little with the

comminution pretreatment (for 30 min), which aimed to increase the accessible area and to reduce the crystallinity and the degree of cellulose polymerization (Zheng et al., 2014; Kratky and Jirout, 2011). After these pretreatments, the cellulose contents were 33.6–37.4%. As described earlier, the reduction in the cellulose content was low with the [Cho][OAc] pretreatment, but it was comparable with that using the other pretreatments.

2.3.2. Effect of [Cho][OAc] IL pretreatment on the enzymatic saccharification of bagasse compared with other pretreatments

Fig.4 shows the time course of the glucose concentration, the saccharification ratio of cellulose, and the glucose yield during the enzymatic saccharification of untreated and pretreated bagasse using various methods to study the effect of [Cho][OAc] IL pretreatment on enzymatic saccharification. After comminution (for 30 min) and alkaline treatment (0.5% NaOH at 121°C for 15 min), the saccharification ratios reached approximately 100% after 72 h. With microwave irradiation (200°C for 10 min), the ratio reached 93.2%, whereas the ratio in untreated bagasse remained 22.6%. These results indicate that these pretreatments were quite effective. However, with [Cho][OAc] pretreatment, the saccharification ratio after 72 h increased with increasing the severity of the conditions, and it reached a maximum value of 98.7% with 130°C for 180 min, while the second highest value of 92.0% was obtained at 110°C for 360 min (97.3% and 98.0% of the total glucose production were saccharified within the first 24 h in these conditions, respectively). The removal of lignin and hemicellulose enhanced the subsequent enzymatic saccharification (Li et al., 2010b), and the increase in the saccharification ratio corresponded to the improvement in the lignin and hemicellulose removal efficiency as the severity increased. However, the saccharification ratio remained 82.7% at 130°C for 60 min, although its composition, i.e., the lignin and hemicellulose contents, was similar to that at 110°C for 360 min and at 130°C for 180 min (Fig.3). It is known that enzymatic saccharification is effected by the cellulose crystallinity, and in general, the anions in ILs function as hydrogen bond

acceptors, which interact with the hydroxyl groups in cellulose to make the crystalline structure of cellulose vulnerable, while the cations interact with lignin via hydrogen bonding and π - π interactions at the same time (Ninomiya et al., 2013c; Wu et al., 2011). Ninomiya et al. (2013a) reported that the [Cho][OAc] also has an effect of reduction in cellulose crystallinity, because the cellulose crystallinity index (CrI) of the microcrystalline cellulose pretreated with [Cho][OAc] at 110°C for 60 min was decreased from 88.5% to 85.1%. However, in the present study, the CrI of bagasse pretreated at 130°C for 180 min was not lower than that of at 130°C for 60 min (the CrI values were 53.1% and 49.3%, respectively). Therefore, the saccharification ratios at 110°C for 360 min and 130°C for 180 min were higher than the ratio at 130°C for 60 min, which may attributed to bare small amount of lignin or other factors which were not become clear in this study.

Thus, [Cho][OAc] pretreatment and the other pretreatments enhanced the saccharification ratio significantly was demonstrated. However, pretreatment causes the loss of lignocellulose components, and the glucose yield after 72 h of saccharification per 1 g of raw bagasse was evaluated (Fig.4). The glucose yields with the comminution (for 30 min), microwave (at 200°C for 15 min), and alkaline (0.5% NaOH at 121°C for 15 min) pretreatments were 0.415, 0.380, and 0.400 g, respectively. With the [Cho][OAc] pretreatment, 0.355 g of glucose per 1 g of raw bagasse at 130°C for 180 min and 0.346 g of glucose per 1 g of raw bagasse at 110°C for 360 min were obtained. Thus, the yields were slightly lower compared with those obtained using the other pretreatments because of the slightly greater loss of the cellulose content after the [Cho][OAc] pretreatment.

2.3.3. Comparison of the EPRs using various pretreatments

A specific consideration of the EPR (Okajima et al., 2014; Khawkomol et al., 2013), which some researchers refer to as the net energy ratio (NER) (Miller et al., 2011), is essential during the production of biomass-derived materials such as biofuels. The EPR (or NER) is defined as the energy output/input ratio, and the production process is worthwhile when $EPR > 1$ (Okajima et al., 2014). In

the present study, the EPR was defined as the energy production/energy requirement during pretreatment for ethanol production, and the EPRs for various pretreatments, including [Cho][OAc] pretreatment were calculated and compared. Based on the specific heat capacity measurement obtained by DSC, the energy required to heat 1 g of [Cho][OAc] from room temperature to 110°C and 130°C were 240 and 279 J, respectively.

Table 1 summarizes the energy productions and requirements and the EPRs for various pretreatments. The maximum energy production was 6.29 kJ/g-raw bagasse using comminution (for 30 min), but the EPR (0.47) was well below 1 because of the high energy requirement (13.50 kJ/g-raw bagasse). However, [Cho][OAc] pretreatment at 110°C for 360 min produced 5.25 kJ/g-raw bagasse, where the production was somewhat lower, but the minimum amount of energy was required (1.30 kJ/g-raw bagasse), thereby yielding the maximum EPR, i.e., 4.04. Thus, the IL pretreatment using [Cho][OAc] was the most energy-saving and cost-effective pretreatment. The energy required for the [Cho][OAc] pretreatment was much lower than that with each of the other pretreatments, which was mainly because of the lower specific heat of [Cho][OAc], e.g. the energy required to heat of [Cho][OAc] from room temperature to 110°C and 130°C was 36% and 31% lower, respectively, compared with that of water, and the small amount of [Cho][OAc] used. However, this requires further study because these values were based on an assumption of an adiabatic state, and it is also necessary to consider the energy requirements of other operations, such as removing the solvent from the pretreated material, as well as optimizing the amounts of solvents used.

2.4. Conclusions

To evaluate the effectiveness of IL pretreatment using [Cho][OAc], the effects of pretreatment on the lignocellulose composition, subsequent enzymatic saccharification, and EPR using various pretreatments, i.e., [Cho][OAc], comminution, microwave irradiation, and alkaline treatment were compared. After 72 h of enzymatic saccharification, 0.355 g of glucose per 1 g of raw bagasse (98.7%

of saccharification ratio) was obtained when the bagasse was pretreated with [Cho][OAc] at 110°C for 360 min, although this was slightly lower than that using the other pretreatments. However, the EPR was 4.04 in this condition, which was the highest, thereby demonstrating that the [Cho][OAc] IL pretreatment was the most energy efficient. These results indicate that IL pretreatment using [Cho][OAc] is very promising for practical applications in the production of useful materials from lignocellulose.

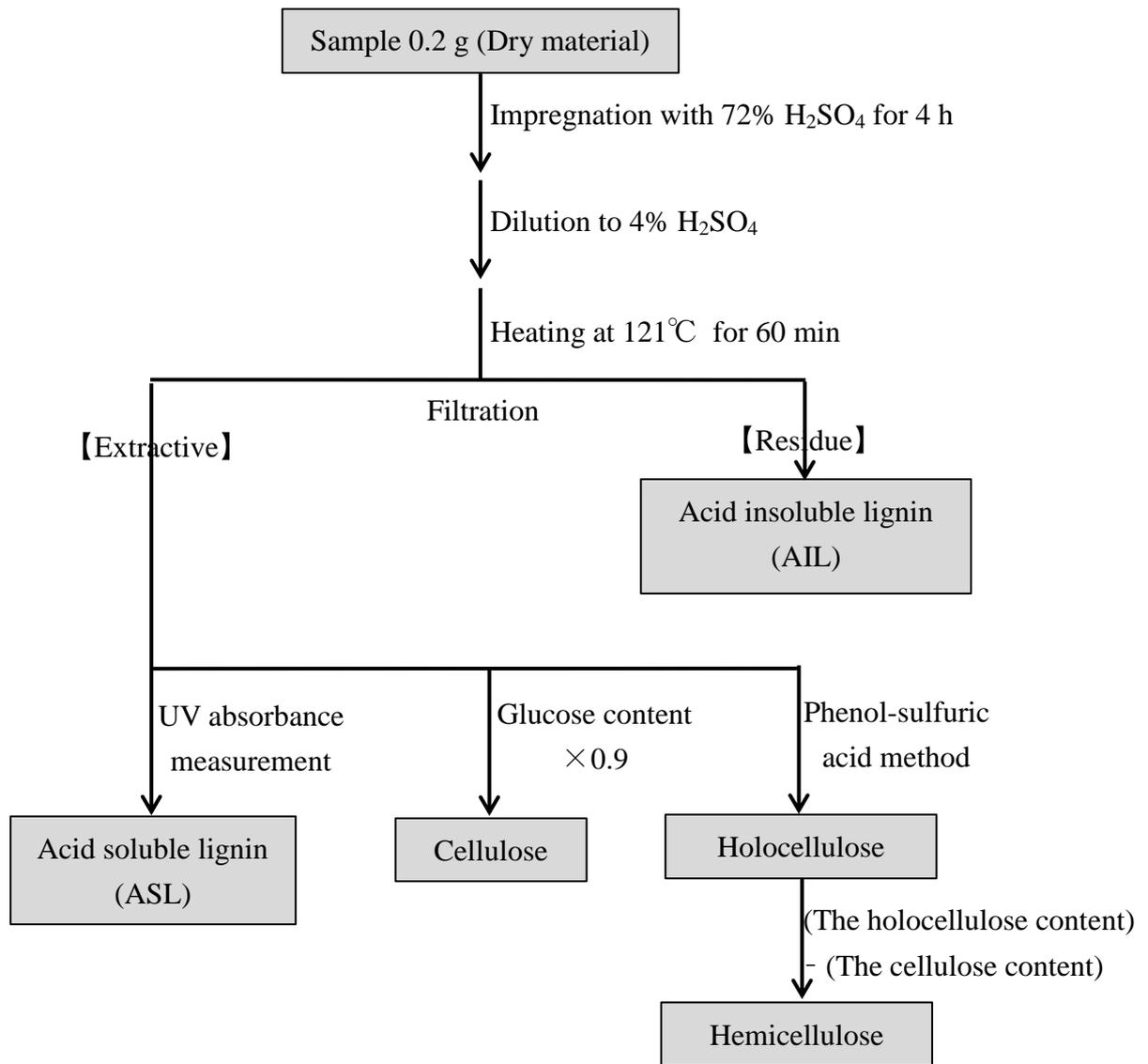


Fig.2 Procedure for analysis of composition

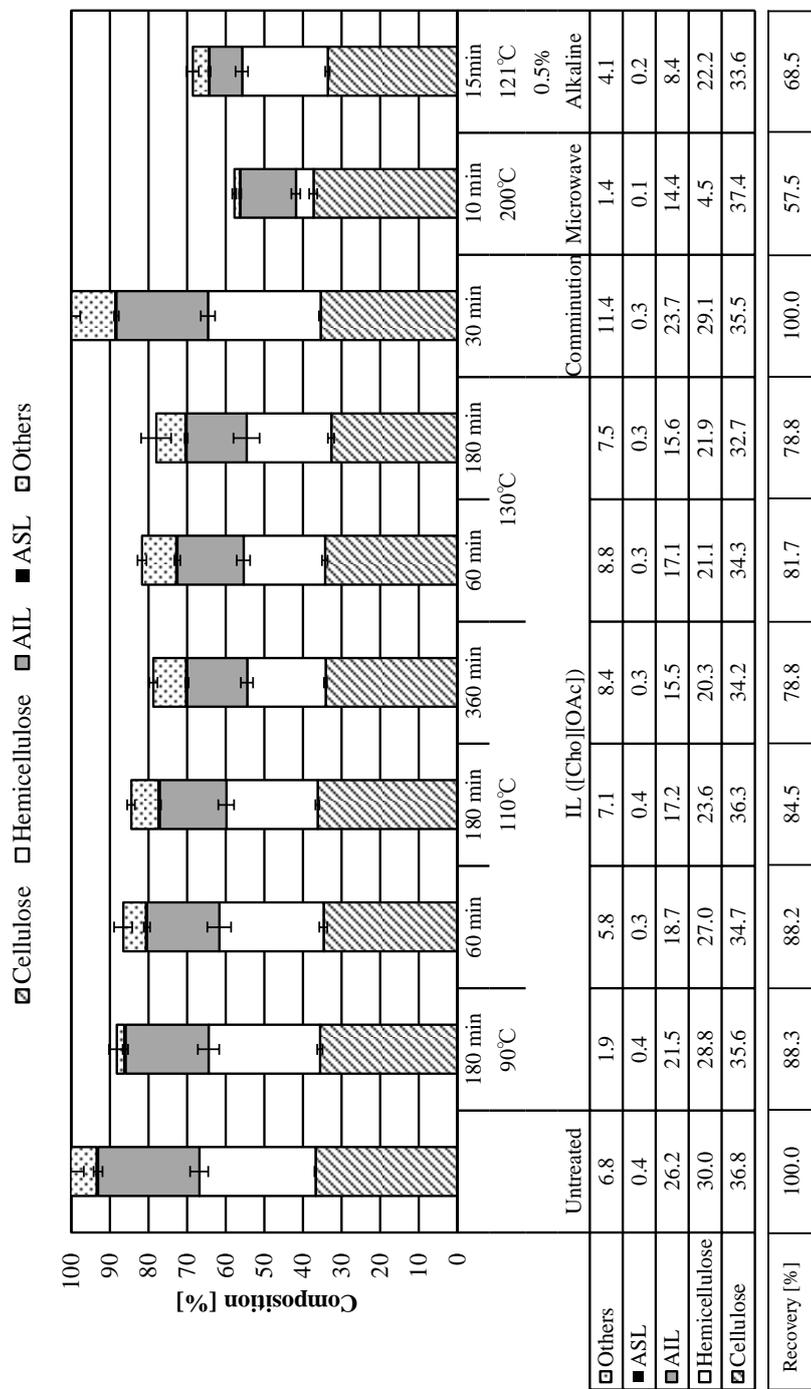
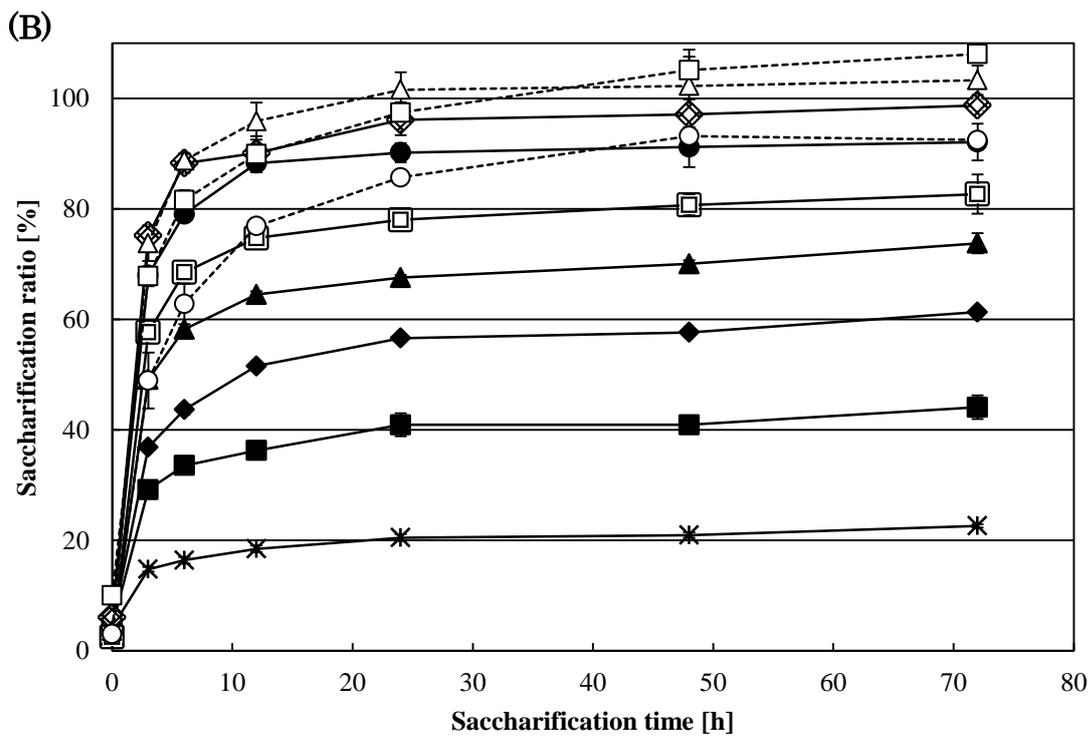
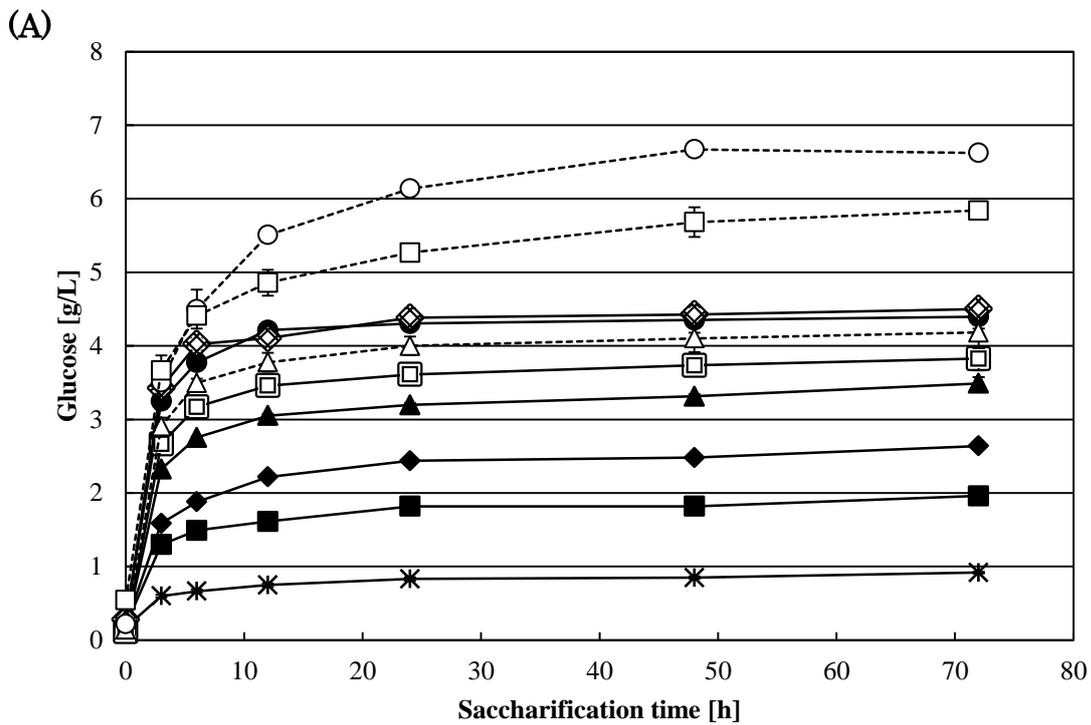


Fig. 3 Compositions of untreated and variously-pretreated bagasse taking account of the gravimetric recovery. The bars indicate the standard deviation from three independent experiments.



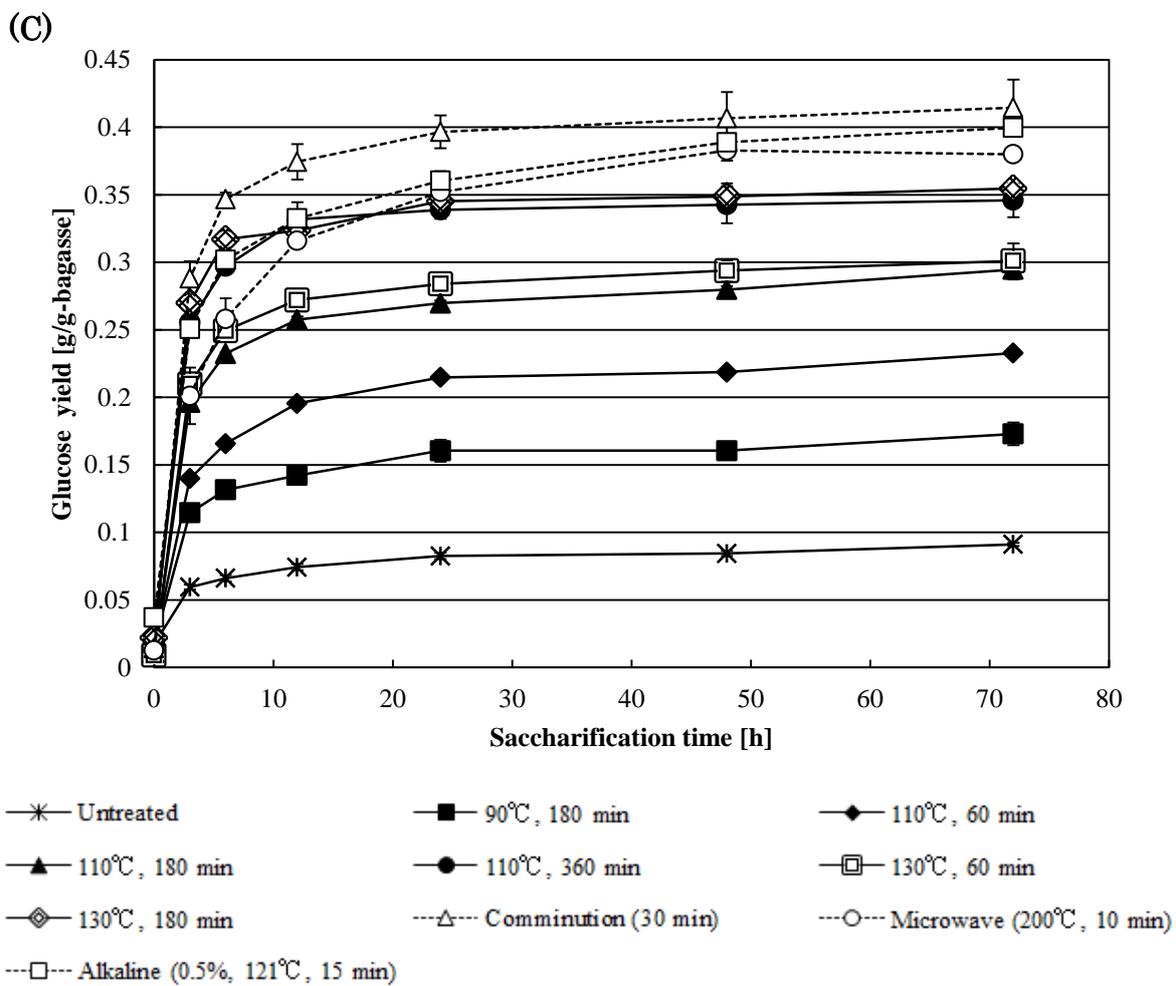


Fig.4 (A) Glucose concentration, (B) saccharification ratio, and (C) glucose yield during the enzymatic saccharification of untreated and variously-pretreated bagasse. The bars indicate the standard deviation from three independent experiments.

Table 1 Comparison of EPRs with various pretreatments.

| | Glucose yield [g/g-bagasse] | Theoretical ethanol yield ^a [g/g-bagasse] | Energy production ^b [kJ/g-bagasse] | Energy requirement ^c [kJ/g-bagasse] | EPR ^d |
|-----------------------------------|--------------------------------|--|---|--|------------------|
| Untreated | 0.091 | 0.046 | 1.38 | 0.45 | 3.07 |
| [Cho][OAc] IL | | | | | |
| (110°C, 360 min) | 0.346 | 0.176 | 5.25 | 1.30 | 4.04 |
| (130°C, 180 min) | 0.355 | 0.181 | 5.38 | 1.45 | 3.72 |
| Comminution (30 min) | 0.415 | 0.211 | 6.29 | 13.50 | 0.47 |
| Microwave (200°C, 15 min) | 0.380 | 0.194 | 5.77 | 14.54 | 0.40 |
| Alkaline (0.5%, 121°C, 15 min) | 0.400 | 0.204 | 6.06 | 8.99 | 0.67 |

a Glucose yield (g/g-bagasse)×0.511

b Theoretical ethanol yield (g/g-bagasse)×29.75 (kJ/g)

c Energy requirement during pretreatment

d Energy production(kJ/g-bagasse)/Energy requirement(kJ/g-bagasse)

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CHAPTER 3. Effect of pretreatment combined cholinium ionic liquid with cosolvent on the enzymatic saccharification of sugarcane bagasse for bioethanol production

1. Introduction

Pretreatment is a requisite step for the production of biofuels or chemicals from lignocellulose through saccharification and fermentation (Mai et al., 2014). Lignocellulose is the complex association of cellulose-lignin-hemicelluloses; it is difficult to break down and offers limited accessibility to enzymes and microorganisms (Auxenfans et al., 2014; Perez-Pimienta et al., 2013). The aim of the pretreatment of lignocellulose is to increase the accessibility of cellulase to cellulose by removing lignin and hemicelluloses, increasing the surface area of cellulose, and decreasing the cellulose crystallinity (Uju et al., 2012; Chang and Holtzapfle, 2000; Yoshida et al., 2008).

Ionic liquid (IL) pretreatment has received much attention since certain types of ILs were discovered to be able to dissolve cellulose (Swatloski et al., 2002). IL is an organic salt, usually composed of an organic cation and an inorganic anion, and existing in liquid form below 100°C (Nasipour et al., 2014; Cheng et al., 2014). ILs have characteristics such as high thermal stability, high ionic conductivity, miscibility, water stability, density, viscosity, polarity, and non-volatility (Vancov et al., 2012; Ninomiya et al. 2013b). Besides, ILs dissolve polar and non-polar organic, inorganic, and polymeric compounds under mild conditions, which means that ILs are capable of dissolving hemicelluloses, lignin, and cellulose in biomass (Ninomiya et al., 2013b; Olivier-Bourbigou et al., 2010).

We used choline acetate ([Cho][OAc]), which is a completely bioderived IL, for pretreatment of lignocellulose. [Cho][OAc] has important advantages, such as being less expensive, more biodegradable, and more biocompatible compared to highly effective ILs such as imidazolium ILs (Ninomiya et al. 2015). In our previous study, we demonstrated that IL pretreatment using [Cho][OAc] at 130°C for 180 min enabled 98.7% of the cellulose of pretreated bagasse to convert into glucose after 72 h of enzymatic saccharification (Asakawa et al, 2015). In addition, the energy profit ratio

(Okajima and Sako, 2014; Khawkomol et al., 2013), which is defined as the energy production/energy requirement during pretreatment for ethanol production, was higher for this pretreatment than for other pretreatments, such as comminution, microwave irradiation, and alkaline treatment. These results indicate that IL pretreatment using [Cho][OAc] is energy-saving and cost-effective, and may be expected to overcome the economic drawbacks of the conventional pretreatment method. However, although [Cho][OAc] is relatively cheap among ILs, [Cho][OAc] is nevertheless expensive as a reagent for pretreatment, and a reduction of cost will be necessary for practical use. Furthermore, the ILs/biomass solution is highly viscous, which makes it difficult to handle and difficult to increase biomass loading (Mood et al., 2013; Fu and Mazza, 2011; Wang et al, 2015). In particular, it is difficult to mix [Cho][OAc] with biomass because of the solid condition of [Cho][OAc] at room temperature.

In order to overcome these problems, this work proposes pretreatment using [Cho][OAc] added to organic solvent. Pretreatments using various organic solvents have been reported, and they have the following advantages: (1) cellulose is recovered as solids with only minor degradation, (2) lignin and hemicelluloses dissolve into organic solvent, which increases the surface area of cellulose, (3) organic solvent can be easily recovered by distillation and recycled (Zhang et al., 2016).

To evaluate the efficiency of pretreatment using [Cho][OAc] with an added organic solvent as cosolvent, hydrolyzabilities of the sugarcane bagasse pretreated with mixtures of [Cho][OAc] and several polar organic solvents were demonstrated. Additionally, componential analysis of [Cho][OAc]/cosolvent-pretreated bagasse, X-ray diffraction (XRD) analysis of the pretreated microcrystalline cellulose, and ethanol fermentation of the pretreated bagasse were performed.

2. Materials and methods

2.1. Materials

The sugarcane bagasse was kindly provided by Kyuyo Sugar Industry (Okinawa, Japan). The raw bagasse was ground by a cutter mill (D3V-10, Osaka Chemical Co., Ltd., Osaka, Japan) for 1 min, and sieved to obtain 100–500 μm fractions, which were then used in all the experiments. The moisture content of the raw bagasse was 3.6%, and the chemical composition were 38.9% cellulose, 28.5% hemicelluloses, 2.5% acid soluble lignin (ASL), 26.4% acid insoluble lignin (AIL), and 3.7% others. Microcrystalline cellulose (Avicel PH-101) and [Cho][OAc] (melting point of 85°C , ≥ 95.0) was obtained from Sigma-Aldrich Japan Co., LLC. (Osaka, Japan). N, N-dimethylacetamide (DMA, >98.0%), N, N-dimethylformamide (DMF, >99.5%), dimethyl sulfoxide (DMSO, >99.0%), N-methyl-2-pyrrolidone (NMP, >97.0%), ethylene glycol (EG, >99.5%), and glycerin (G, 99.0%) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The commercial enzyme mixture, Meicelase (derived from *Trichoderma viride*; 224 FPU/g: b-glucosidase activity, 264 IU/g) was provided by Meiji Seika Pharma Co., Ltd (Tokyo, Japan). In the SSF experiments, *Saccharomyces cerevisiae* BA11 (Bio Academia CO., Ltd., Osaka, Japan) was used as an ethanol production strain.

2.2. Pretreatment combining [Cho][OAc] IL with cosolvent

1.5 g of the bagasse or Avicel was placed in a 300-ml eggplant flask with 4.5 g of [Cho][OAc]/cosolvent (1:1 w/w) and incubated in an oil bath at 130°C for 180 min. The contents of the flask were mixed at 30 min after the beginning of heating and then every 60 min until the end of the heating period. Following incubation, 135 ml of distilled water was added, and the residue was separated from the mixture by filtration, which was then washed thoroughly four times with an equal volume of distilled water. The residue on the filter paper was collected and stored at 4°C for subsequent enzymatic saccharification. For compositional analysis and recovery measurement, the pretreatment was conducted as described above, and the residue after washing was dried overnight at $105^{\circ}\text{C} \pm 3^{\circ}\text{C}$.

2.3. Componential analysis

The acid-soluble lignin (ASL), acid-insoluble lignin (AIL), cellulose, and hemicellulose contents of pretreated bagasse were determined as follows. 3 ml of 72% (w/w) H₂SO₄ was added to 0.2 g of the sample, and the solution was left at room temperature for 4 h. The mixture was then diluted to 4% (w/w) H₂SO₄ and autoclaved at 121°C for 60 min. The ASL content was determined from the UV absorbance of this hydrolyzed solution at 205 nm using an absorption coefficient of 110 L g⁻¹ cm⁻¹. The cellulose content was determined based on the monomer content (glucose) in the hydrolyzate. The glucose content was determined using HPLC equipment with a refractive index detector (RID-10A, Shimadzu Co., Ltd., Kyoto, Japan) with an Aminex column (HPX-87H; Bio-Rad, Richmond, U.S.A.) at 65°C, with 5.0 mM H₂SO₄ as the mobile phase at 0.6 ml/min. The hemicellulose content was determined by subtracting the cellulose content from the holocellulose content. The holocellulose content was determined using the phenol-sulfuric acid method. The insoluble residue in the hydrolysate, i.e., AIL (high molecular weight lignin), was washed, dried to constant weight at 105°C ± 3°C, and weighed. All the analytical determinations were performed in triplicate, and the means were calculated.

2.4. X-ray diffraction (XRD) analysis

The XRD patterns of the treated Avicel were obtained using the X-ray diffractometer (Multiflex; RIGAKU, Yamanashi, Japan). The samples were scanned in the 2θ range of 5-45° with the step size of 0.2° at 40 kV and 40 mA at a temperature of 25°C. The crystallinity index (CrI) was determined by multiple peak separation method. Crystalline peak ($2\theta=22.1-22.5^\circ$) and amorphous peak ($2\theta=20.2-21.0^\circ$) was separated using the software package JADE7 (MDI, Livermore, USA) and RaspWin (HT Soft Lab, Kuala Lumpur, Malaysia), and the CrI was calculated by the following equations:

$$\text{CrI (\%)} = (\text{Sc} / \text{St}) \times 100$$

Where, Sc and St were the crystalline area and the total area (the crystalline area + the amorphous area), respectively.

2.5. Enzymatic saccharification

Enzymatic saccharification of untreated and pretreated bagasse was performed in a 110-mL vial containing 10 mL of 100 mM sodium acetate buffer (pH 5.0), 0.2 g of the sample, and 0.02 g of enzyme (Meicelase). The reaction condition was at 50°C on a rotary shaker at 140 rpm for 72 h. The glucose concentration was determined at specific time intervals using an enzymatic determination glucose assay kit (Autokit Glucose, Wako Pure Chemical Industries Ltd., Osaka, Japan). The saccharification ratio and glucose yield were calculated using the following equations:

$$\text{Saccharification ratio (\%)} = \{ \text{Glucose produced (g/L)} \times 0.9 \} / \{ \text{Cellulose in pretreated sample (g/L)} \} \times 100$$

$$\text{Glucose yield (g/g-bagasse)} = \text{Glucose produced (g)} / \text{Raw bagasse (g)}$$

All the enzymatic saccharification experiments were performed in triplicate, and the means were calculated.

2.6. Simultaneous saccharification and fermentation (SSF)

S. cerevisiae BA11 was incubated on potato dextrose agar plates at 30°C and then stored at 4°C. Pure yeast culture from an agar plate was added to 10 mL tubes containing 5 mL of the sterilized preculture medium. It contained 10 g/L glucose, 1 g/L yeast extract, 0.1 g/L KH₂PO₄, 0.1 g/L MgSO₄ · 7H₂O and

0.1 g/L (NH₄)SO₄ (Itoh et al., 2003). The preculture was incubated at 30°C for 24 h using a seesaw incubator at 60 rpm. 1.0 g of the pretreated bagasse in a 200-mL Erlenmeyer flask was autoclaved at 121°C for 20 min, and then 50 mL of the sterilized mainculture medium and 0.1 g of Meicelase were added. The precultured yeast suspension was centrifuged, the supernatant was removed, and the suspended yeast by sterilized water was added. The main culture medium contained 2 g/L yeast extract, 1 g/L (NH₄)₂HPO₄, 0.05 g/L MgSO₄·7H₂O and 50 mM sodium acetate buffer (pH 5.0). The mixture was incubated in a rotary shaker at 40°C and 100 rpm. Glucose and ethanol concentration were determined by HPLC equipment with a refractive index detector (RID-10A, Shimdzu Co., Ltd.) with an Aminex column (HPX-87H; Bio-Rad) at 65°C, with 5.0 mM H₂SO₄ as the mobile phase at 0.6 ml/min. The ethanol conversion ratio was calculated using the following equation:

$$\text{Ethanol conversion ratio (\%)} = \frac{\{\text{Ethanol produced (g/L)}\}}{\{\text{Cellulose in pretreated sample (g/L)} \times 1.1 \times 0.51\}} \times 100$$

The SSF experiments were performed in triplicate, and the means were calculated.

3. Results and discussion

3.1. Effect of pretreatment with combined [Cho][OAc] and cosolvent on enzymatic saccharification of bagasse

To evaluate the combination use of [Cho][OAc] and various organic solvents such as DMA, DMF, DMSO, NMP, EG, and G, the bagasse pretreated with [Cho][OAc]/cosolvents was enzymatically hydrolyzed. Since the organic solvents used in this work have high boiling points, it is not necessary to use pressure-resistant equipment and evaporation at 130°C of the pretreatment temperature. DMA, DMF, and DMSO are aprotic polar solvents, and the addition of these solvents to an imidazolium-IL enhances the cellulose dissolution at 25°C, as observed by Xu et al. (2013). NMP is an aprotic polar

solvent as with DMA, DMF, and DMSO. On the other hand, EG and G are protic polar solvents, and the addition of a protic polar solvent to IL leads to decline in cellulose solubility, as also pointed out by Xu et al. (2013). However, EG and G have the advantage of a low toxicity, and it has been reported that they have been independently used in the pretreatment of biomass (Alriols et al., 2009; Sun et al., 2015). Therefore, they were employed in this work by way of comparison to aprotic polar solvents.

The glucose concentration, saccharification ratio, and glucose yield after 72 h of the enzymatic saccharification of the untreated, [Cho][OAc]-pretreated, and [Cho][OAc]/cosolvents-pretreated bagasse are summarized in Table 2. The saccharification ratio of the [Cho][OAc]-treated bagasse reached 91.0%, a significant increase compared to 26.2% for the untreated bagasse. Ninomiya et al. (2013b) reported a similar result, showing that pretreatment with [Cho][OAc] at 110°C for 16 h increased the saccharification ratio of kenaf powder from less than 20% up to almost 100% after 48-h enzymatic saccharification. This enhancement effect of [Cho][OAc] pretreatment on enzymatic saccharification might be explained by the different roles of cations and anions in IL. The former interacts with lignin through hydrogen bonding and π - π interactions, and the latter acts as hydrogen bond acceptors that interact with the hydroxyl group of cellulose (Ninomiya et al. 2013b), leading to the decline of the cellulose crystallinity, hemicelluloses and lignin content, and the increase of surface area. As a result, the enzymatic saccharification kinetics and the yield of fermentable sugars are improved (Sun et al., 2013).

Subsequently, pretreatment using [Cho][OAc] and solvents with a weight ratio of 1:1 enhanced the saccharification ratio of the treated bagasse to 59.4–97.4%, especially the DMSO (97.4%), NMP (91.6%), and EG (90.7%). In these cases, the saccharification ratios and the glucose yields are approximately equal to those from [Cho][OAc] treatment alone. [Cho][OAc]/DMSO, [Cho][OAc]/NMP, and [Cho][OAc]/EG were 0.392 g, 0.400 g, 0.393 g, and 0.393 g, respectively. A similar result was seen in the combination use of imidazolium-IL and DMSO, which enhanced the enzymatic saccharification of rice straw, as reported by Mai et al. (2014). According to the above

results, the addition of the cosolvents such as DMSO, NMP, and EG into [Cho][OAc] was able to reduce the amount of IL used by 50%, was revealed.

3.2. Influence of [Cho][OAc]/cosolvents pretreatment on cellulose

As described above, the pretreatment combined use of [Cho][OAc] and cosolvents such as DMSO, NMP, and EG promoted the enzymatic saccharification of bagasse approximately equal to the single use of [Cho][OAc] in spite of 50% reduction of the amount of IL used. In general, the enhancement effect on the enzymatic saccharification of IL pretreatment is attributed to an increase in the accessibility of cellulase to cellulose, which is caused by the synergetic effect of the reduction in cellulose crystallinity, and the removal of lignin and hemicelluloses (Uju et al., 2012; Sun et al., 2013). To reveal the structural changes of bagasse by the enhancement effect on the enzymatic saccharification of pretreatment with [Cho][OAc]/cosolvents, we first investigated this pretreatment's influence on cellulose.

Fig. 5 shows the recovery ratio and CrI of the untreated, the [Cho][OAc]-treated, and the [Cho][OAc]/cosolvent-treated Avicel. A significant decrease was observed in the ratio of CrI of Avicel after the [Cho][OAc] treatment, from 81.6% to 55.4%. Ninomiya et al. (2013a) also reported that CrI of the Avicel treated with [Cho][OAc] at 110°C for 60 min decreased from 88.5% to 85.1%. The greater effect on CrI decrease in the present work can be attributed to the severity of pretreatment condition. The reduction in cellulose crystallinity with IL pretreatment is explained as follows. The anion in IL first attacks the free hydroxyl groups on cellulose and deprotonates them, while the cation interacts with the hydroxyl oxygen atoms. The hydrogen bonds in cellulose are disrupted and replaced by hydrogen bonding between the IL anions and cellulose hydroxyls, resulting in cellulose dissolution and disruption of the crystalline structure (Qiu et al., 2012). After IL pretreatment, regenerated cellulose becomes amorphous and porous, thereby improving the accessibility of cellulase (Mood et al., 2013; Dadi et al. 2006). On the other hand, 18.0% of the Avicel was lost after the [Cho][OAc]

treatment. This can be explained by the fact that part of the Avicel fragmented into soluble oligosaccharides that could not regenerate by addition of water, and therefore were lost (Qiu et al, 2012; Zhu et al, 2012; Nguyen et al., 2010).

The recovery ratios and CrIs of Avicel after the [Cho][OAc]/cosolvents treatments were 73.9%–87.5% and 61.6%–76.3%, respectively. This showed that the [Cho][OAc]/cosolvents treatments also had the effect of reduction in cellulose crystallinity, however, the effect were lower than with the [Cho][OAc] treatment. However, this result was not related to the result of the enzymatic saccharification. For example, the [Cho][OAc]/EG treatment caused the high saccharification ratio and the high glucose yield of the enzymatic saccharification equal to the single use of [Cho][OAc], however, its CrI (76.3%) was the highest of all pretreatments. EG is a protic polar solvent as with G, and it was reported that an addition of a protic polar solvent to an imidazolium-IL reduced the cellulose dissolution into the solution by preferential solvation of anions which play an important role in cellulose dissolution (Xu et al., 2013). The higher recovery ratio and CrI obtained by not only the [Cho][OAc]/EG treatment but also the [Cho][OAc]/G treatment, which is probably explained by the description by Xu et al.

On the other hand, it was reported that the addition of an aprotic polar solvent such as DMA, DMF, and DMSO to an imidazolium-IL enhanced the cellulose dissolution (Mai et al., 2014; Xu et al., 2013). An aprotic polar solvent serves to solvate cations and promotes IL dissociation into solvated cations and “free” anions, resulting in enhanced cellulose dissolution (Xu et al., 2013). However, reduction in CrI by the treatment using [Cho][OAc] combined with an aprotic polar solvent such as DMA, DMF, DMSO, and NMP was not observed in this study. Because it was reported that the addition of cosolvents at a high ratio reduced the hydrogen-bond basicity of ILs/cosolvents (Mai et al., 2014), the addition ratio of DMSO to [Cho][OAc] in the [Cho][OAc]/DMSO pretreatment, which is the one of the most effective treatment in glucose yield of enzymatic saccharification, varied between 7:3, 1:1, and 3:7. As shown in Fig. 5, CrI of Avicel increased along with the DMSO ratio (CrIs of

[Cho][OAc]/DMSO at ratios of 1:0 ([Cho][OAc] alone), 7:3, 1:1, and 3:7 were 55.4%, 61.3%, 66.2% and 67.2%, respectively), whereas the recovery ratio rather decreased (the recovery ratios were 82.0%, 78.3%, 79.6% and 75.0%, respectively). This means that the addition of DMSO do not promote an effect of the [Cho][OAc] treatment on the reduction in cellulose crystallinity, rather than as an addition ratio of DMSO increases, the effect decreases. The result which is different from the literatures may be caused by properties of ILs, experimental condition, or other factors. Besides, the difference in recovery ratio and CrI by the difference of the cosolvents was not related to the cosolvents' property (e.g. dielectric constant, dipole moment), and the cause was not clarified. Further research on this point is necessary in the future, too.

According to the above results, it revealed that all the [Cho][OAc]/cosolvents tested in this study had an enhancement effect on the reduction in cellulose crystallinity, although the effect was lower than with the [Cho][OAc] pretreatment alone.

3.3. Influence of [Cho][OAc]/cosolvents pretreatment on the chemical composition of bagasse

As we know, the removal of lignin and hemicelluloses enhances the enzymatic saccharification by making cellulose more accessible to cellulase (Zhu et al., 2012). To investigate the influence of the [Cho][OAc]/cosolvents pretreatment on the chemical composition of bagasse, composition analysis of untreated, [Cho][OAc]-pretreated, and [Cho][OAc]/cosolvents-pretreated bagasse was conducted. Fig. 6 shows the chemical composition, and the each content was calculated by multiplying the content based on the pretreated bagasse as 100% by the recovery ratio. After the [Cho][OAc] pretreatment, an obvious decrease in hemicelluloses (21.6%) and AIL (18.5%) was observed, although there was little decrease of cellulose (37.4%). This change of the composition is attributed to the interaction with IL and biomass as mentioned in section 3.1., and the result means that the [Cho][OAc] pretreatment can be considered an efficient method due to removal of lignin and hemicelluloses and the high recovery of cellulose.

With the pretreatment using [Cho][OAc]/cosolvents except DMSO, the recovery ratios (61.7–80.6%) were lower than or nearly equal to that of the [Cho][OAc] treatment. In these cases, hemicelluloses (14.4–19.8%) and AIL (9.0–14.5%) contents were somewhat decreased compared to the use of [Cho][OAc] alone. In the [Cho][OAc]/DMSO treatment, the recovery ratio and the composition was similar to the [Cho][OAc] treatment. This means that the removal effect of hemicelluloses and AIL by the [Cho][OAc]/cosolvent treatment was equal to or higher than the [Cho][OAc] treatment alone. For this reason, the high saccharification ratio of the enzymatic saccharification might be obtained from the bagasse pretreated with [Cho][OAc]/cosolvents. On removal of hemicelluloses and lignin, it is known that the treatment with organic solvent at high temperatures and pressures for a specific time causes the degradation of a majority of lignin and hemicelluloses into small molecular weight fragments (Zhang et al., 2016). The enhancement of removal of hemicelluloses and AIL in these [Cho][OAc]/cosolvents treatments might be caused by the property of cosolvent. However, Mai et al. (2014) reported that DMA and DMF did not dissolve, while DMSO dissolved large amount of hemicelluloses and lignin at 80°C. In this work, the effect of removal hemicelluloses and lignin were DMA>DMF>DMSO, and the result does not accord with the property of these organic solvents reported by Mai et al. (2014). On The cellulose content (33.6-35.7%) of the [Cho][OAc] treatments added DMA, DMF, NMP, and G were slightly lower compared to the [Cho][OAc] treatment alone (37.4%), except DMSO (38.3%) and EG (29.4%). No relation between the result and the recovery ratio and CrI of the [Cho][OAc]/cosolvents-treated Avicel was observed, which might be caused the presence of other components such as hemicelluloses and lignin interacting with IL/cosolvents, and other factors.

As described above, the difference in the composition of bagasse after the [Cho][OAc]/cosolvents pretreatments could not be completely explained by the cosolvents' property to dissolve the individual components. It seemed that the chemical composition of bagasse treated with [Cho][OAc]/cosolvents was influenced by various factors in a complex manner.

3.4. Influence of the [Cho][OAc]/DMSO ratio on enzymatic saccharification

As described previously, the pretreatment using [Cho][OAc] added DMSO, NMP, and EG with weight ratio of 1:1 enhanced the saccharification ratio of bagasse, and its glucose yields were approximately equal to the [Cho][OAc] treatment. The result suggested that the addition of the cosolvent might be able to reduce the production cost of useful materials such as ethanol.

The highest glucose yield among the tested mixtures was obtained from the [Cho][OAc]/DMSO (1:1 w/w) treatment, therefore, the influence of the [Cho][OAc]/DMSO ratio on the enzymatic saccharification was investigated. Table 2 shows the result after 72 h of the enzymatic saccharification of bagasse pretreated with the [Cho][OAc]/DMSO mixture at [Cho][OAc]/DMSO ratios of 1:0 ([Cho][OAc] alone), 7:3, 1:1, 3:7, and 0:1 (DMSO alone). The saccharification ratio of [Cho][OAc] alone was 91.0%. The saccharification ratio increased along with the DMSO ratio, reaching a maximum value of 97.4% at a [Cho][OAc]/DMSO ratio of 1:1. From that point onward, the saccharification ratio decreased, and treatment using DMSO alone hardly enhanced the saccharification ratio (29.2%). Except for the [Cho][OAc]/DMSO ratio of 7:3, whose glucose yield was lower owing to the lower recovery (data not shown), a similar tendency was seen in the glucose yield, and a maximum glucose yield of 0.400 g/g-raw bagasse, which were equal to that of [Cho][OAc] pretreatment alone, was obtained with a [Cho][OAc]/DMSO ratio of 1:1. Furthermore, 0.365 g/g of the glucose yield, corresponding to more than 90% of that of the single use of [Cho][OAc], was obtained at a [Cho][OAc]/DMSO ratio of 3:7 albeit 70% reduction of [Cho][OAc]. These results indicated that the combination use with DMSO could reduce the amount of [Cho][OAc] used by 50% or more. Further research from the view point of environmentally sustainability could focus on recycle of [Cho][OAc] and cosolvent.

3.5. Fermentability of bagasse pretreated with [Cho][OAc]/DMSO

To evaluate the fermentability of bagasse pretreated with a [Cho][OAc]/DMSO mixture, which may include residual [Cho][OAc] and DMSO, SSF of ethanol using *S. cerevisiae* BA11 was performed. The time course of glucose and ethanol concentrations in the SSF using 20 g/L of bagasse pretreated with [Cho][OAc]/DMSO (1:1 w/w) as a substrate is shown in Fig. 7. The ethanol concentration was rapidly increased after starting the incubation, and an ethanol concentration of more than 70% was produced in first 6 h. Thereafter, the ethanol concentration increased slowly and achieved a maximum of 3.7 g/L after 48 h of incubation, which corresponded to the ethanol conversion ratio of 69.9%. On the other hand, most of the glucose was not detected from beginning to end, due to the ethanol conversion rate being higher compared to the glucose production rate. These results indicate that the ethanol fermentation of the [Cho][OAc]/DMSO-pretreated bagasse could be performed without obvious inhibitions. However, since Asada et al. (2015) reported that 74% of ethanol conversion was obtained in the SSF of steam-exploded cedar, the lower ethanol conversion rate in this work seems to be improved by optimizing the fermentation condition. This area is ripe for further research.

4. Conclusions

The pretreatment combining [Cho][OAc] with polar organic solvents was attempted. The glucose yield after 72 h of enzymatic saccharification of bagasse treated with [Cho][OAc] and a cosolvent such as DMSO, NMP, or EG at weight ratio of 1:1 were approximately equal to those of [Cho][OAc] alone, and 50% reduction of the amount of [Cho][OAc] was achieved. It was revealed that these pretreatments had the lower effect on reduction in cellulose crystallinity, however, the equal or higher effect on removal hemicelluloses and lignin compared to the single use of [Cho][OAc]. The [Cho][OAc]/cosolvent pretreatment can be a promising method to overcome the drawback of the high cost of IL pretreatment was suggested.

Table 2 After 72 h of enzymatic saccharification of untreated, [Cho][OAc]-pretreated, and [Cho][OAc]/cosolvents-pretreated bagasse.

| Sample | Glucose [g/L] | Saccharification ratio [%] | Glucose yield [g/g-raw bagasse] |
|---------------------|---------------|----------------------------|------------------------------------|
| Untreated | 1.81 ± 0.04 | 26.2 ± 0.5 | 0.091 ± 0.002 |
| [Cho][OAc] Alone | 9.61 ± 0.40 | 91.0 ± 3.8 | 0.392 ± 0.020 |
| [Cho][OAc]:DMA=1:1 | 9.29 ± 0.29 | 86.3 ± 3.1 | 0.341 ± 0.017 |
| [Cho][OAc]:DMF=1:1 | 8.34 ± 0.12 | 80.8 ± 1.9 | 0.357 ± 0.010 |
| [Cho][OAc]:DMSO=1:1 | 10.28 ± 0.17 | 97.4 ± 1.3 | 0.400 ± 0.007 |
| [Cho][OAc]:NMP=1:1 | 10.03 ± 0.38 | 91.6 ± 4.0 | 0.393 ± 0.022 |
| [Cho][OAc]:EG=1:1 | 9.56 ± 0.38 | 90.7 ± 3.6 | 0.393 ± 0.019 |
| [Cho][OAc]:G=1:1 | 6.43 ± 0.62 | 59.4 ± 5.6 | 0.236 ± 0.022 |

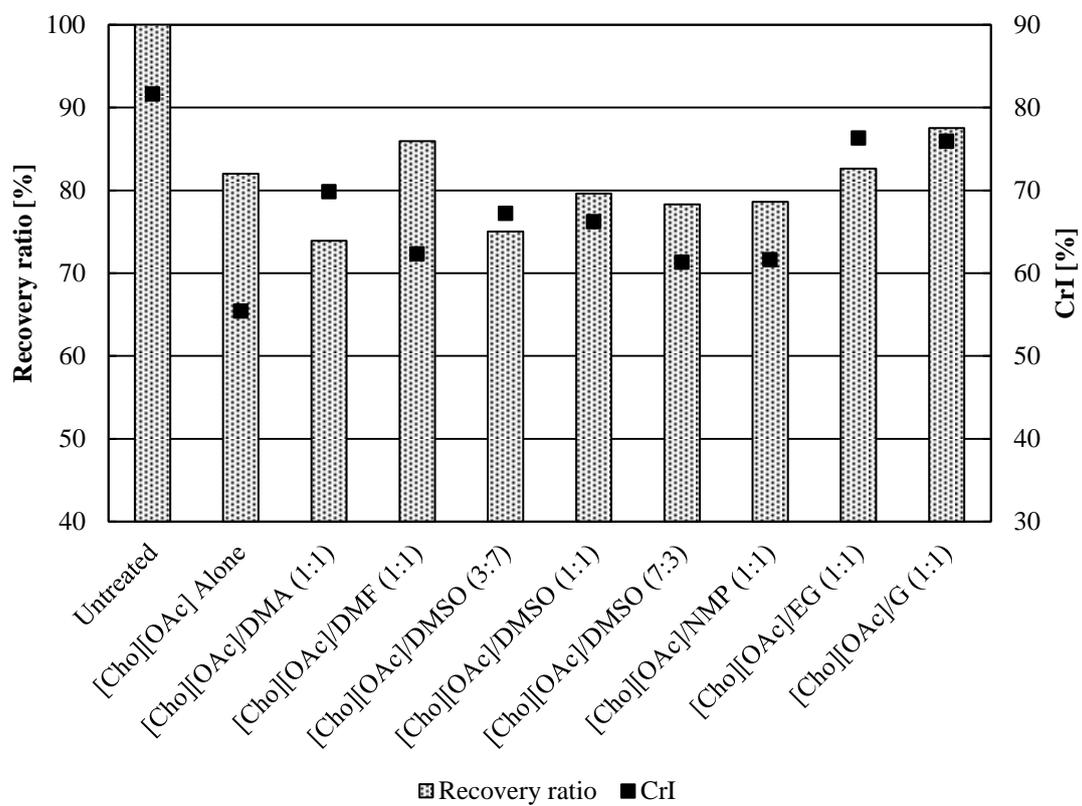


Fig.5 Recovery ratio and CrI of Avicel after pretreatment with [Cho][OAc]/cosolvents.

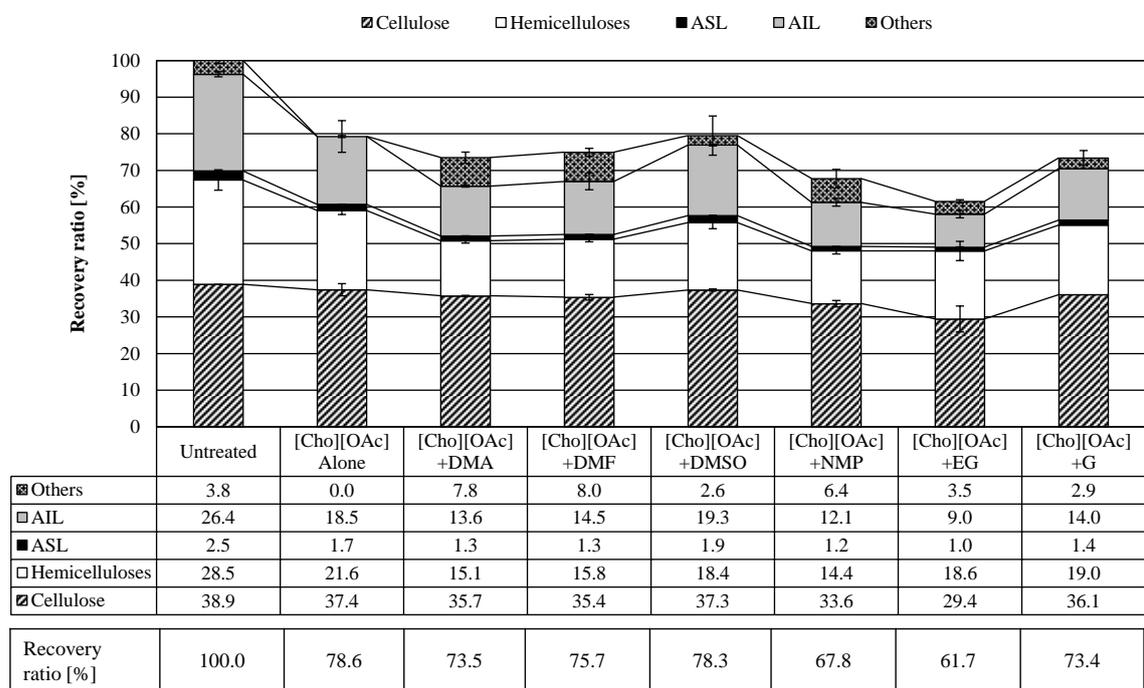


Fig.6 Composition of untreated and pretreated-[Cho][OAc]/cosolvents (1:1 w/w) bagasse.

Table 3 After 72 h of enzymatic saccharification of bagasse pretreated with [Cho][OAc]/DMSO mixture with various [Cho][OAc]/DMSO ratios.

| Sample | Glucose [g/L] | Saccharification ratio [%] | Glucose yield [g/g-raw bagasse] |
|---|---------------|----------------------------|------------------------------------|
| [Cho][OAc]:DMSO=1:0 ([Cho][OAc] Alone) | 11.17 ± 0.47 | 91.0 ± 3.8 | 0.392 ± 0.020 |
| [Cho][OAc]:DMSO=7:3 | 11.17 ± 0.18 | 93.3 ± 2.6 | 0.384 ± 0.011 |
| [Cho][OAc]:DMSO=1:1 | 10.28 ± 0.17 | 97.4 ± 1.3 | 0.400 ± 0.007 |
| [Cho][OAc]:DMSO=3:7 | 9.03 ± 0.91 | 88.4 ± 8.1 | 0.365 ± 0.033 |
| [Cho][OAc]:DMSO=0:1 (DMSO Alone) | 2.58 ± 0.41 | 29.2 ± 4.5 | 0.100 ± 0.016 |

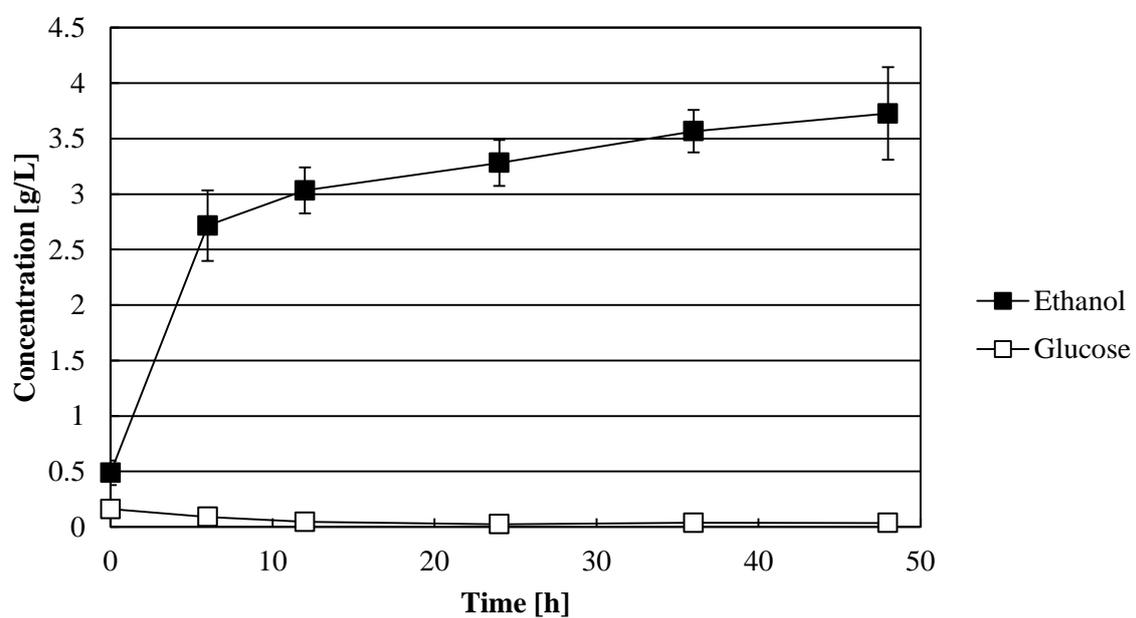


Fig.7 Time course of SSF production of [Cho][OAc]/DMSO-pretreated bagasse to ethanol. The bars indicate the standard deviation from three independent experiments.

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CONCLUSIONS

In this study, a novel ionic liquid (IL) pretreatment using choline acetate ([Cho][OAc]) was proposed. First, to evaluate the effectiveness of the [Cho][OAc] pretreatment, the effect of the pretreatment was compared with that of various pretreatments, i.e., comminution, microwave irradiation, and alkaline treatment. After 72 h of enzymatic saccharification, 0.355 g of glucose per 1 g of raw bagasse (98.7% of saccharification ratio) was obtained when the bagasse was pretreated with [Cho][OAc] at 110°C for 360 min. In this condition, 4.04 of the maximum EPR was obtained, thereby demonstrating that the [Cho][OAc] IL pretreatment was the most energy efficient.

Secondly, the pretreatment combined use of [Cho][OAc] and polar organic solvents was attempted. The glucose yield after 72 h of enzymatic saccharification of bagasse treated with [Cho][OAc] and a cosolvent such as DMSO, NMP, or EG at weight ratio of 1:1 were approximately equal to those of [Cho][OAc] alone, and 50% reduction of the amount of [Cho][OAc] was achieved.

These results indicated that the [Cho][OAc] pretreatment was energy efficient, and the combination use of cosolvent might be able to reduce the high cost of IL, therefore it is a very promising method for practical applications in the production of useful substances from lignocellulosic biomass.

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