Saccharification and ethanol fermentation from cholinium ionic liquid-pretreated bagasse with a different number of post-pretreatment washings

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Abstract

Choline acetate (ChOAc), a cholinium ionic liquid (IL), was compared with 1-ethyl-3-methylimidazolium acetate (EmimOAc) with regard to biomass pretreatment, inhibition on cellulase and yeast, residuals in pretreated biomass, and saccharification and fermentation of pretreated biomass. Irrespective of ChOAc and EmimOAc, cellulose and hemicellulose saccharification of the IL-pretreated bagasse were over 90% and 60%, respectively. Median effective concentrations (EC_{50}) based on cellulase activity were 32 wt% and 16 wt% for ChOAc and EmimOAc, respectively. The EC_{50} based on yeast growth were 3.1 wt% and 0.3 wt% for ChOAc and EmimOAc respectively. The residuals in IL-pretreated bagasse were 10% and 23% for ChOAc and EmimOAc, respectively, when washed 2 times after pretreatment. Ethanol yield on a bagasse basis were 60% and 24% for ChOAc and EmimOAc, respectively, in the saccharification and fermentation of IL-pretreated bagasse when washed 2 times. ChOAc-pretreated bagasse could be saccharified and fermented with fewer wash times than EmimOAc-pretreated bagasse.

Keywords:

Cholinium, Ionic liquid, Pretreatment, Lignocellulose, Wash times

1. Introduction

In this decade, ionic liquids (ILs; generally defined as organic salts that melt below 100°C) have been the focus of considerable attention owing to their thermal stability, non-volatility, and capability of dissolving various polymeric compounds. It was previously demonstrated that some ILs can dissolve cellulose (Swatloski et al., 2002) and that the re-precipitated cellulose showed a higher efficiency of enzymatic saccharification because the crystallinity was significantly decreased (Dadi et al., 2006). This IL-assisted pretreatment has been applied toward several types of lignocellulosic materials (Brandt et al., 2013), since the IL-assisted pretreatment has been demonstrated to be more effective than the conventional pretreatment using diluted acid or ammonia (Li et al., 2010, 2011).

After the IL-assisted pretreatment step, the pretreated biomass needs to be washed extensively to remove the residual IL since the residual IL in pretreated biomass inhibits cellulolytic enzymes and fermentative microbes in the subsequent saccharification and fermentation step (Datta et al., 2010; Ouellet et al., 2011). This extensive washing of pretreated biomass results in large amounts of diluted IL aqueous solution, which leads to high costs for both concentrating IL from its diluted aqueous solution by evaporation and treating the resultant wastewater.

Recently, completely bio-derived cholinium ILs were reported, which contain either cholinium cations combined with amino acid-based anions (Hu et al., 2007) or carboxylic acid-based anions (Fukaya et al., 2007). Moreover, it was demonstrated that these cholinium ILs could be used for biomass pretreatment to enhance the enzymatic saccharification of lignocellulosic biomass (Liu et al., 2012; Ninomiya et al., 2013a,b,c, 2014, 2015a,b), as conventional imidazolium IL. Furthermore, our previous study

demonstrated that cholinium IL was less inhibitory to cellulase enzymes and fermentative microorganisms than imidazolium IL (Ninomiya et al., 2015, 2013a). However, to the best of our knowledge, even for the imidazolium IL, there are few papers reporting the effect of the number of washings on the residual IL amount in the pretreated biomass and the resulting inhibition of enzymatic saccharification of the IL-pretreated biomass, (Li et al., 2009; Li et al., 2013), and there are no papers reporting the above points for the cholinium IL.

Therefore, the present study investigated the effect of the number of washings on the amount of residual cholinium IL in pretreated biomass and the resulting inhibition toward enzymatic saccharification and microbial fermentation of the cholinium IL-pretreated biomass. Moreover, cholinium IL was also examined with regard to biomass pretreatment capability and inhibitory effect on cellulolytic enzymes and fermentation microorganisms. Specifically, choline acetate (ChOAc) was used as the biocompatible cholinium IL. The obtained data for the pretreatment capability, the inhibitory effect on cellulolytic enzymes and fermentation microorganisms, the residual IL amount, the enzymatic saccharification, and the microbial fermentation were compared with data obtained using 1-ethyl-3-methylimidazolium acetate (EmimOAc), the conventional imidazolium IL employed for biomass pretreatment.

2. Materials and Methods

2.1. Biomass, IL, cellulase, and yeast cells

Bagasse powder (approximately 3 mm) was purchased from Toyota Motor Corporation (Miyoshi, Japan) as the lignocellulosic material. The bagasse powder was ground by a mill and then sieved to obtain a powder of <250 μm . Microcrystalline

cellulose (Avicel[®] PH-101) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Phosphoric acid swollen cellulose (PASC) was prepared from the microcrystalline cellulose according to the method described previously (Wood, 1988). ChOAc was prepared by using a one-pot neutralization method with minor modifications (Yu et al., 2008). EmimOAc was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Commercial cellulase (Cellic[®] CTec2), a complex blend of cellulase, hemicellulose, and β -glucosidase, (batch number VCNI 0008, 106 filter paper units (FPU) per milliliter) was obtained from Novozymes Japan, Ltd (Chiba, Japan). FPU of the cellulase was determined by the method reported by NREL (Adney and Baker, 2008). The yeast strain *Saccharomyces cerevisiae* MT8-1 (Tajima et al., 1985) was used for ethanol fermentation. All other chemicals were obtained from commercial sources and were of reagent grade.

2.2. Assay of inhibition by IL on cellulase and yeast activity

For evaluating the inhibitory effects of IL to cellulase activity, enzymatic saccharification was conducted in a 30 mL glass vial containing 5 mL of a mixture of 50 mM phosphate buffer (pH 5.0), IL (EmimOAc or ChOAc) at final concentrations of 0%–50% (w/w), CTec2 at a concentration of 40 FPU/g of dry biomass, and 0.03 g of dry weight equivalent of wet PASC as the model substrate. The vial was incubated at 50°C for 48 h using a reciprocal shaker at 130 rpm. A sample was taken at 48 h and then heated at 90°C for 5 min to inactivate the enzymes. The heated sample was then centrifuged at $21,500 \times g$ for 1 min and the supernatant analyzed for glucose by high performance liquid chromatography (HPLC) as described below. The response to the ILs were evaluated based on the relative cellulase activity, which was defined as the

percentage of the generated glucose at 48 h in the presence of the IL relative to the generated glucose at 48 h of a control culture free from the IL. The median effective concentration based on cellulase activity (cEC_{50}) was determined as the IL concentration at which the relative cellulase activity was reduced to 50%.

For evaluating the inhibitory effects of IL to yeast, the culture was conducted in a 5-mL test tube containing autoclaved YPD medium (10 g/L of yeast extract, 20 g/L of Bacto-peptone, and 20 g/L of glucose; Nacalai Tesque, Kyoto, Japan) supplemented with the IL (EmimOAc or ChOAc) at final concentrations of 0.3%–5% (w/w). The yeast cells were precultured aerobically at 30°C in the test tube containing 5 mL of YPD medium free from IL. The precultured broth was transferred into a test tube at an initial optical density of 0.1 at a wavelength of 600 nm (OD_{600}). The test tube was incubated at 30°C for 48 h using a reciprocal shaker at 160 rpm. The culture broth was sampled at specified culture time points and the OD_{600} was measured. The response to the IL was evaluated based on the relative growth, which was defined as the percentage of the OD_{600} at 48 h in the presence of the IL relative to the OD_{600} at 48 h of a control culture free from the IL. The median effective concentration based on yeast growth (yEC_{50}) was determined as the IL concentration at which the relative growth was reduced to 50%.

2.3. IL-assisted pretreatment and washing of biomass

For biomass pretreatment, 0.5 g of bagasse powder was added to a 15-mL polypropylene tube (Corning Inc., New York, United States) containing 5 g of IL (EmimOAc or ChOAc). After vortexing, the biomass/IL mixture in the tube was sonicated for 60 min at 24 kHz and an emission power of 35 W using an ultrasonic processor (UP200S with sonotrode S3, Hielscher Ultrasonics GmbH, Teltow, Germany)

in a water bath maintained at 25°C.

For washing the IL from the pretreated biomass, 45 mL of deionized water was then added to the bagasse/IL mixture in a 50-mL tube, which resulted in the precipitation of the biomass. After mixing with inversion for 3 min, the 50-mL tube was centrifuged ($8000 \times g$) for 10 min at 25°C and the supernatant was removed. The washing procedure was repeated 1–5 times. For respective washing procedures, the removed supernatant was subjected to measurement of IL concentration using HPLC. The amount of residual IL in the biomass was indirectly determined from the IL amount in the supernatant. The precipitated wet bagasse was stored at 4°C until use for subsequent enzymatic saccharification.

2.4. Saccharification and subsequent fermentation

Saccharification was performed in a 50-mL polypropylene tube (Corning Inc.) containing the wet pretreated biomass (0.25 g as dry weight equivalent) mixed with 5 mL of 50 mM phosphate buffer (pH 5.0) and CTec2 at a concentration of 40 FPU/g of dry biomass, which resulted in about 3.3% solid loading. The amount of wet biomass loading was determined from the moisture content of the pretreated biomass with sufficient washing. The tube was set in a heat block (Thermo Block Rotator SN-06BN; Nissinrika Co., Tokyo, Japan) and axially rotated at 35 rpm for 48 h under a controlled temperature of 50°C. After enzymatic saccharification for 48 h, the 50-mL tube was centrifuged ($8000 \times g$) for 10 min at 4°C. The liquefied portion (6 mL) was sampled and used for sugar quantification and subsequent fermentation. For sugar analysis, 1 mL of the sample was heated at 90°C for 5 min to inactivate the enzymes. After centrifugation of the heated sample at $21,500 \times g$ for 1 min, the supernatant was subjected to glucose

and xylose measurements, as described below. Cellulose saccharification was evaluated as the percentage of cellulose hydrolyzed into glucose compared with cellulose in the original bagasse. In the same manner, hemicellulose saccharification was evaluated as the percentage of hemicellulose hydrolyzed into xylose compared with hemicellulose in the original bagasse.

For fermentation, the yeast was aerobically precultured for 24 hours at 30°C and 135 rpm in a 500-mL Sakaguchi flask containing 100 mL of YPD medium. The yeast cells were collected by centrifugation at 3000 rpm for 5 min and washed thrice with saline. Fermentation was then performed in a 30-mL glass vial equipped with a gas check valve, which contained a mixture of the 4 mL of sugar solution obtained in the saccharification step, 1 mL of quintuple strength YPD medium, and the yeast suspension at a final concentration of 100 g of wet suspension/L. During the fermentation, samples were collected after 12, 24, and 48 h. After centrifugation of the samples at $21,500 \times g$ for 1 min, the supernatant was subjected to the ethanol measurement as described below. Ethanol yield on a glucose basis was evaluated as the percentage of ethanol compared with the ethanol theoretically converted from glucose obtained by saccharification. In the same manner, ethanol yield on a bagasse basis was evaluated as the percentage of ethanol compared with the ethanol theoretically converted from cellulose in the original bagasse.

2.5. Analysis of biomass composition

The cellulose, hemicellulose, and lignin contents of the original bagasse were determined according to the NREL method (Sluiter et al., 2012) with minor modifications. In brief, 0.1 g of the sample was mixed with 2 mL of a 72% (v/v) H₂SO₄

aqueous solution for 2 h at RT. The mixture was transferred to a 200-mL Erlenmeyer flask, diluted with 75 mL of water, and autoclaved at 121°C for 15 min. The acid-diluted hydrolysate was filtered, after which the amount of acid-insoluble lignin was gravimetrically determined by measuring the residue on the filter after drying at 100°C for 12 h. The amount of acid-soluble lignin was determined from the ultraviolet (UV) absorbance of the filtrate at 205 nm and an absorption coefficient of $110 \text{ L g}^{-1} \text{ cm}^{-1}$ ([TAPPI, 1991](#)). The sum of the acid-insoluble and acid-soluble lignin was regarded as the total amount of lignin. The amount of sugars in the hydrolysate was determined by HPLC as described below. The amount of cellulose and hemicellulose was calculated from the glucose and xylose content multiplied by the anhydro correction factors of 162/180 and 132/150 respectively.

2.6. Analysis of glucose, xylose, ethanol, and IL concentrations

Glucose and xylose concentrations were determined by HPLC equipped with a refractive index (RI) detector (Shimadzu Co., Kyoto, Japan) and a CARBOSep CHO-682 column (Tokyo Chemical Industry Co., Ltd, Tokyo, Japan). The column was run at 85°C with a water mobile phase and a flow rate of 0.4 mL/min. The ethanol concentrations were determined using gas chromatography (GC) equipped with a flame ionization detector (FID) (Shimadzu, Kyoto, Japan) and a CBP-20-S25-050 capillary column (0.32 mm ID, 25 m, Shimadzu, Kyoto, Japan). The injector temperature was 250°C, and the FID temperature was 280°C. The column temperature was maintained at 40°C for 5 min. Nitrogen was used as the carrier gas at 40 mL/min. ChOAc and EmimOAc concentrations were determined using HPLC equipped with a refractive index (RI) detector (Shimadzu Co., Kyoto, Japan) and an ICSep-ION-300 column

(Tokyo Chemical Industry Co., Ltd, Tokyo, Japan). The column was run at 75°C with a mobile phase of 4.25 mM H₂SO₄ and a flow rate of 0.4 mL/min.

3. Results and discussion

3.1. Pretreatment capability of ChOAc or EmimOAc for enzymatic saccharification of bagasse

To examine the pretreatment capability of ChOAc or EmimOAc for subsequent enzymatic hydrolysis of lignocellulosic biomass, bagasse powder was pretreated with each IL and then hydrolyzed by Ctec2 cellulase for 48 h. Here the pretreated bagasse was washed 5 times to sufficiently remove the IL before saccharification so as to eliminate any inhibitory effect of IL on the cellulolytic enzymes. Especially, in the case of EmimOAc-pretreated bagasse, the gel-like structure of EmimOAc-pretreated bagasse was mashed carefully using a spatula in the washing procedure. Table 1 lists the saccharification percentage of ChOAc- or EmimOAc-pretreated bagasse at 48 h. Without IL pretreatment, the cellulose and hemicellulose saccharification percentages were 24% and 9% of the theoretical maximum at 48 h. In contrast, with ChOAc pretreatment, cellulose and hemicellulose saccharification percentages were 90.3% and 99.9% at 48 h. These values are approximately the same as those using EmimOAc. These results indicate that ChOAc had a sufficient pretreatment capability for lignocellulosic materials, which was at almost the same level as EmimOAc under the examined pretreatment condition.

EmimOAc has frequently been considered as the most effective IL for cellulose dissolution and pretreatment of enzymatic hydrolysis (Brandt et al., 2013). In general, anions act as hydrogen bond acceptors that interact with the hydroxyl group of cellulose,

thereby weakening the crystalline structure of cellulose (Remsing et al., 2006). Among the anions of imidazolium ILs, acetate anion has been reported to be the highest hydrogen bond basicity (β) and capability of reducing cellulose crystallinity (Brandt et al., 2013). This is why the pretreatment with ChOAc also enhanced the saccharification of cellulose and hemicellulose to the same degree as the pretreatment with EmimOAc (Table 1). In our previous study, X-ray diffraction (XRD) analysis had been conducted to examine the cellulose crystallinity in the bagasse powder pretreated in ChOAc or EmimOAc with ultrasound irradiation, and a crystallinity index (CrI) was determined on the basis of the XRD spectra. The CrI values were 61.9%, 49.1%, and 41.7% for the original biomass, biomass pretreated in ChOAc, and biomass pretreated in EmimOAc, respectively (Ninomiya et al., 2015b). These results are also generally consistent with the cellulose saccharification results shown in Table 1, suggesting that ChOAc had a substantial pretreatment capability for lignocellulosic biomass. The reason why the saccharification percentage and the CrI values for ChOAc were slightly lower than those for EmimOAc is possibly because the imidazolium cation uniquely interacts with lignin through hydrogen bonding and π - π interactions (Janesko, 2011), which results in the delignification of biomass via lignin depolymerization (Sun et al., 2014). Moreover, our previous study had also revealed that the composition was almost the same between untreated bagasse powder and bagasse powder pretreated in ChOAc or EmimOAc with ultrasound irradiation under the condition employed in this study (Ninomiya et al., 2015b). Therefore, the pretreatment method used in this study could enhance subsequent saccharification not due to change in biomass composition, but mainly due to change in crystalline structure of cellulose.

3.2. Inhibitory effect of ChOAc or EmimOAc on cellulolytic enzymes and yeast cells

To examine the inhibitory effect of ChOAc or EmimOAc on cellulase enzymes, enzymatic saccharification of the PASC as amorphous cellulose was conducted for 24 h using Ctec2 cellulase in the presence of the prescribed concentration of each IL. The cEC_{50} values were determined from the dose–response relationships between the relative enzyme activity and the IL concentration (Fig. S1A). The cEC_{50} values were 32 wt% and 16 wt% for ChOAc and EmimOAc, respectively (Table 1). To examine the inhibitory effect of ChOAc or EmimOAc on yeast cells, *S. cerevisiae* MT8-1 was cultured for 48 h in the presence of the prescribed concentration of each IL. The yEC_{50} values were determined from the dose–response relationships between relative yeast growth and the IL concentration (Fig. S1B). The yEC_{50} values were 3.1 wt% and 0.3 wt% for ChOAc and EmimOAc, respectively (Table 1). These results indicate that ChOAc shows less inhibitory effect on cellulase and yeast activity compared with EmimOAc under the examined conditions.

Biocatalysts such as enzymes and microbes in IL aqueous solution generally lose their activity due to a higher salt concentration and ionic strength. Thus, a more biocompatible IL was needed to perform the saccharification of IL-pretreated biomass with fewer post-pretreatment washings. The inhibitory effects of ChOAc on cellulase and yeast cells were significantly lower than those of EmimOAc (Table 1) because the cholinium cation was reported to be more intrinsically biocompatible than the imidazolium cation (Ninomiya, 2015, 2013b; Petkovic et al., 2010; Hou et al., 2013).

3.3. Residual amount of IL in ChOAc or EmimOAc-pretreated biomass with a different number of washings

The morphology of biomass was observed after the IL-pretreatment followed by washing the prescribed number of times. [Figure S2B-E](#) shows the bagasse powder, which was pretreated with EmimOAc or ChOAc and then washed once or 5 times. In the case of EmimOAc, pretreated bagasse exhibited a gel-like morphology irrespective of the number of wash times, 1 or 5 ([Fig. S2B and D](#)). On the other hand, in the case of ChOAc, pretreated bagasse exhibited a powdered morphology ([Fig. S2C and E](#)), like non-treated bagasse ([Fig. S2A](#)). It was considered that these different morphologies for EmimOAc and ChOAc-pretreated bagasse would lead to differences in washing efficiency and differences in the residual amount of IL in the IL-pretreated biomass.

To examine the amount of residual IL in the ChOAc or EmimOAc-pretreated biomass, the bagasse was pretreated with each IL and then washed 1–5 times. [Figure 1](#) shows the residual IL percentage in pretreated biomass with a different number of post-pretreatment wash times. Irrespective of the ILs, the percentages decreased as the number of washing times increased, reaching the approximate plateaus at 5 washings. The residual IL percentage in the case of ChOAc was lower than the cases for EmimOAc throughout the number of wash times. In the case of EmimOAc, the residual IL percentages were 46% and 9% at 1 and 5 wash times, respectively. On the other hand, in the case of ChOAc, the residual IL percentages were 20% and 4% at 1 and 5 wash times, respectively. These results indicate that ChOAc was more easily washed away from the pretreated bagasse compared than EmimOAc.

The biomass-derived gel is actually generated when antisolvent (water or alcohol) is added into the biomass/IL solution to re-precipitate the biomass once it is solubilized into IL (Dibble et al., 2011). The biomass-derived gel is so stiff that it cannot be completely dispersed in the antisolvent and the IL cannot be easily separated from the

biomass-gel, which causes a loss of the expensive IL and an inhibition toward cellulolytic enzymes and fermentation microorganisms in the subsequent processes. In the present study, it was found that ChOAc generated less biomass-gel and less IL remained in the pretreated bagasse compared with that for EmimOAc-treated biomass (Fig. 1). This may be because EmimOAc has a relatively higher capability to reduce the cellulose crystallinity and to swell the biomass than ChOAc. The residual IL percentage was 9% at 5 wash times in the case of EmimOAc, whereas the residual IL percentage was reduced to the same level with only 2 wash times in the case of ChOAc (Fig. 1). This means that pretreatment with ChOAc can reduce the amount of wastewater.

3.4. Saccharification and subsequent fermentation of ChOAc or EmimOAc-pretreated biomass with a different number of wash times

To examine the saccharification efficiency of ChOAc or EmimOAc-pretreated biomass with a different the number of post-pretreatment wash times, the pretreated bagasse powders were washed 1–5 times, and then subjected to enzymatic saccharification using Ctec2 for 48 h. Figure 2A and B show the cellulose and hemicellulose saccharification percentages obtained at 48 h of saccharification of ChOAc or EmimOAc-pretreated bagasse with a different number of wash times. The saccharification percentages increased as the number of washing times increased, irrespective of the ILs. The saccharification percentages for the case of ChOAc were higher than those for the cases of EmimOAc throughout the 5 wash times. In the case of EmimOAc, the cellulose and hemicellulose percentages were 73% and 33% at 5 wash times, respectively. Whereas in the case of ChOAc, cellulose, and hemicellulose, the saccharification percentage was 88% and 66% at 5 wash times, respectively. Even at 2

wash times, the cellulose and hemicellulose saccharification percentages for ChOAc reached 75% and 43%, respectively, although the cellulose and hemicellulose saccharification percentages were just 44% and 5%, respectively, in the case of EmimOAc.

To examine the fermentation efficiency of sugars saccharified from ChOAc or EmimOAc-pretreated biomass with a different number of post-pretreatment wash times, the pretreated bagasse powder, which was washed 1–5 times, was enzymatically hydrolyzed for 48 h, followed by ethanol fermentation using yeast MT8-1 for another 48 h. Figure 2C shows ethanol yield on a glucose basis obtained at 48 h of fermentation from glucose generated by saccharification of ChOAc or EmimOAc-pretreated bagasse with a different number of wash times. The ethanol yield enhanced along with the number of washing times, reaching saturated values, irrespective of the ILs. The ethanol yield for the case of ChOAc was higher than those for the cases of EmimOAc at 1 and 2 wash times. The ethanol yields at 2 wash times were 73% and 54% in the cases of ChOAc and EmimOAc, respectively. The ethanol yields were almost at the constant level of around 75% irrespective of the ILs at 3–5 wash times.

Figure 3 shows the overall ethanol yield on the original bagasse basis obtained at 48 h of fermentation from glucose generated by saccharification of ChOAc or EmimOAc-pretreated bagasse with a different number of wash times. As can be expected from the results in Figure 3, the overall ethanol yield enhanced along with the number of washing times, reaching the saturated values at approximately 60%, irrespective of the ILs. The ethanol yield for the case of ChOAc was higher than those for the cases of EmimOAc at 1–3 wash times. The overall ethanol yields at 2 wash times were 60% and 24% for the case of ChOAc and EmimOAc, respectively. Four

post-pretreatment washing times were required to reach the saturated value in the case of EmimOA. These results indicate that ChOAc-pretreated bagasse could be saccharified and fermented with fewer wash times than EmimOAc-pretreated bagasse.

The present study, for the first time, compared cholinium IL (ChOAc) and imidazolium IL (EmimOAc) systematically from the viewpoint of the pretreatment capability of biomass (Table 1), the inhibitory effects on cellulase and yeast (Table 1), the residual percentage in pretreated biomass after post-pretreatment washing (Fig. 1), and the saccharification and subsequent fermentation (Figs. 2 and 3). It was revealed that ChOAc-pretreated bagasse could be saccharified and fermented with fewer wash times than EmimOAc-pretreated bagasse (Figs. 2 and 3). This was because the ChOAc showed almost the same capability of biomass pretreatment for saccharification (Table 1), the less inhibitory effects on cellulase and yeast (Table 1), and the less residual amounts in the pretreated bagasse due to less biomass-derived gel (Fig. 1). We therefore suggest that ChOAc is a more favorable IL for biomass pretreatment prior to saccharification and fermentation since it can reduce the number of post-pretreatment wash times and reduce the resultant wastewater of diluted IL aqueous solution under the examined conditions.

Figure 4 shows the dose–response relationships for cellulose saccharification percentage, hemicellulose saccharification percentage and ethanol yield on a glucose basis, which were plotted against the residual IL concentration in the enzymatic reaction mixture and yeast culture media. This Fig. 4 was obtained by the combined use of the data shown in Figs. 2 and 1E. It confirmed that ChOAc shows less inhibitory effect on cellulose and hemicellulose saccharification with Ctec2 cellulase than EmimOAc (Fig. 4A and B). These dose–response profiles for Ctec2 cellulase activity were more

sensitive than those shown in Figure S1A, since the substrate was not PASC but IL-treated bagasse. On the other hand, the dose–response profiles for ethanol yield on a glucose basis overlapped between the cases of ChOAc and EmimOAc (Fig. 4C), although ChOAc showed less inhibitory effect on yeast growth activity than EmimOAc (Fig. S1B). This is because the inoculum size was larger in the case of ethanol fermentation (initial $OD_{600} = 10$) compared with the case of the growth inhibition assay (initial $OD_{600} = 0.1$). Above all, we suggest that ChOAc is a promising alternative to EmimOAc for the saccharification and fermentation of IL-pretreated biomass with fewer post-pretreatment washing and less wastewater, although the positive effect of ChOAc was mainly seen in saccharification under the examined conditions in the present study.

Based on the techno-economic analysis for the bioethanol production process based on IL-pretreatment, enzymatic saccharification, and yeast fermentation (Klein-marcuschamer et al., 2011), it is necessary to attain the high IL recovery ratio (over 97%) and high biomass loading to IL (33–50 wt%) during the pretreatment step as well as the low IL cost (less than \$2.5/kg) to ensure the minimum ethanol selling price (MESP) at \$5/gal. As for the high IL recovery ratio, our previous study (Ninomiya et al., 2015a) demonstrated that the recovery ratio of the ionic liquid (ChOAc) was about 95% and its pretreatment capability was kept during 5 cycles of recycle. As for the high biomass loading to IL during the pretreatment step, our previous study (Ninomiya et al., 2013b) demonstrated that ChOAc pretreatment at a biomass loading up to 25 wt% did not deteriorate pretreatment efficiency (i.e., cellulose saccharification), but the pretreatment efficiency decreased to less than half when biomass loading was increased to 50 wt%. Therefore, it is important to attain the sufficient pretreatment efficiency by a

combined use of ChOAc and microwave irradiation and so on, even when the biomass loading is 50 wt%. Reducing both the ChOAc amount for biomass pretreatment and the number of washing times after pretreatment will make the IL-assisted bioethanol production process more economically and environmentally friendly because of less IL use and less wastewater discharge.

4. Conclusions

The present study compared ChOAc and EmimOAc from the viewpoint of the pretreatment capability of biomass, the inhibitory effects on cellulase and yeast, the residual percentage in pretreated bagasse after post-pretreatment washing, and the saccharification and subsequent fermentation of pretreated bagasse. The ChOAc showed almost the same capability of pretreatment for saccharification, less residual IL in the pretreated bagasse, and less inhibitory effects on cellulase and yeast. As a result, ChOAc-pretreated bagasse could be saccharified and fermented even with fewer wash times than EmimOAc-pretreated bagasse. This could reduce the wastewater from the diluted IL aqueous solution.

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Figure captions

Fig. 1 Percentages of residual IL in IL-pretreated bagasse with different number of wash times. Open bars: EmimOAc, and closed bars: ChOAc. The error bars indicate the standard error from three independent experiments.

Fig. 2 (A) Cellulose saccharification percentage and (B) hemicellulose saccharification percentage obtained using data from the 48-h saccharification of IL-pretreated bagasse with different number of wash times. The 100% cellulose and hemicellulose saccharification corresponds to 13.8 g/L glucose and 8.6 g/L xylose, respectively. (C) Ethanol yield on a saccharified glucose basis. The yield was obtained using data from the 48-h fermentation of glucose saccharified from IL-pretreated bagasse with different number of wash times. Open bars: EmimOAc, and closed bars: ChOAc. The error bars indicate the standard error from three independent experiments.

Fig. 3 Ethanol yield on an original bagasse basis. The yield was obtained using data from the 48-h fermentation of glucose saccharified from IL-pretreated bagasse with different number of wash times. The 100% ethanol yield corresponds to 5.6 g/L ethanol. Open bars: EmimOAc, and closed bars: ChOAc. The error bars indicate the standard error from three independent experiments.

Fig. 4 Correlation plots of (A) cellulose saccharification, (B) hemicellulose saccharification, and (C) ethanol yield on a saccharified glucose basis against

residual IL concentration, which were obtained for different number of wash times. Open keys: EmimOAc, and closed keys: ChOAc. The error bars indicate the standard error from three independent experiments.

Table 1. Pretreatment capability and inhibitory effect of EmimOAc or ChOAc.

IL	Pretreatment capability of IL on bagasse powder ^a		Inhibitory effect of IL on cellulase and yeast ^b	
	Cellulose saccharification percentage [%]	Hemicellulose saccharification percentage [%]	cEC ₅₀ [wt%]	yEC ₅₀ [wt%]
EmimOAc	102.3 ± 0.3	104.7 ± 4.0	16.2 ± 0.3	0.3 ± 0.01
ChOAc	90.3 ± 5.0	99.9 ± 6.2	32.3 ± 1.3	3.1 ± 0.11

The data were expressed as mean ± standard error from three independent experiments.

a Pretreatment of bagasse powder was carried out by sonication in each IL at 25°C for 60 min. The cellulose and hemicellulose saccharification percentage was obtained at 48 h of enzymatic reaction of pretreated bagasse with sufficient post-pretreatment wash.

b The cEC₅₀ value was determined based on cellulase activity of Ctec2 using PASC as substrate. The yEC₅₀ value was determined based on growth activity of yeast *S. cerevisiae* MT8-1.

Fig. 1

Fig. 2

Fig. 3

Fig. 4