Pretreatment of Pussy Willow and Korean Pine Using Various Ionic Liquids and their Mixtures with Organic Solvents for Enzymatic Saccharification

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Different ionic liquids (ILs) and their mixtures with organic solvents (OSs) were investigated to determine their effects on pretreatment for enzymatic saccharification of pussy willow and Korean pine. Combinations of three ILs, namely (1-ethyl-3-methylimidazolium acetate ([EMIM]Ac), 1-butyl-3-methylimidazolium bromide ([BMIM]Br), and 1,3-dimethylimidazolium methyl sulfate ([MMIM]$MeSO_4$)) and three OSs (N,N-dimethylformamide (DMF), N,N-dimethylacetamide (DMAc), and dimethyl sulfoxide (DMSO)) were used. Acremonium cellulase and Optimash BG were used for enzymatic saccharification. The viscosity of ILs and their mixtures with OSs was reduced by adding and increasing the OS amount in mixtures. The viscosity of [BMIM]Br was considerably decreased by the addition of OSs. For both species, the water-soluble fractions (WSFs) obtained using pure ILs decreased with increasing OS content in mixtures. For all co-solvent systems, the WSFs for pussy willow were twice as large as those for Korean pine. For both species, the yields of glucose and xylose were higher for [EMIM]Ac and its mixtures with OSs than for [BMIM]Br, [MMIM]$MeSO_4$, and their mixtures. However, only small differences between glycan and xylose yields among the products pretreated by [BMIM]Br, [MMIM]$MeSO_4$, and their mixtures were observed. The overall xylose yields for pussy willow were higher than those for Korean pine for all pretreatments because xylan is the main hemicellulose component of hardwood.

Keywords: Pretreatment; Ionic liquid; Organic solvent; Pussy willow; Korean pine

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INTRODUCTION

Recently, pretreatment of lignocellulosic biomass using ionic liquids (ILs) as green solvents has attracted substantial attention because of advantages such as easy recovery, chemical stability, temperature stability, non-flammability, low vapor pressure, and wide range of available liquids (Cvjetko Bubalo *et al*. 2015; Halder *et al*. 2019; Han *et al*. 2020a). Despite their many advantages, several drawbacks of the pretreatment using ILs must be addressed. For example, the viscosity of 1-butyl-3-methylimidazolium chloride ([BMIM]Cl) is as high as 142 mPa·s at 80 °C, and the IL is found in the solid state at room temperature (Fendt *et al*. 2011). Thus, its dissolution capability is usually achieved at high temperatures that may lead to unstable IL properties, unwanted side reactions, and loss of treated biomass. Weerachanchai *et al*. (2012) reported that a significant loss of biomass

yield resulted from the pretreatment with ILs ([EMIM]Ac, [MMIM]$MeSO_4$, 1-ethyl-3-methylimidazolium diethyl phosphate ([EMIM]$DePO_4$)) at high temperatures (150 and 180 °C) due to the excessive degradation of carbohydrates including monosaccharides, oligosaccharides, furfural, and 5-hydroxymethylfurfural (HMF) into the WSF at high temperatures.

Several studies on the pretreatments using ILs have suggested the use of co-solvent systems with OSs to compensate the shortcomings of IL-based pretreatments. (Han *et al*. 2020b). The co-solvents would also enhance their solvating capability because these co-solvents could improve mass transportation by decreasing the solvent viscosity without having a significant influence on the specific interactions between cations and anions or between ILs and biomass components, especially cellulose (Andanson *et al*. 2014; Zhang *et al*. 2017). Wu *et al*. (2013) reported on the use of a co-solvent system of [EMIM]Ac and DMSO with different mixing ratios for the pretreatment of eucalyptus wood in order to improve the enzymatic saccharification yield. An appropriate ratio of [EMIM]Ac and DMSO can minimize [EMIM]Ac consumption while maintaining the pretreatment performance due to the viscosity reduction effect obtained by DMSO addition. Weerachanchai and Lee (2013) reported on a decrease in the viscosity of ILs ([BMIM]Cl and [EMIM]Ac) obtained by mixing the ILs with DMF, DMAc, DMSO, and ethanolamine that have high Hildebrand solubility parameters in order to increase the pretreatment capability for corncob and rice straw. The pretreatment using [EMIM]Ac and its mixture with DMAc (40 to 60%) provided similar sugar conversion yield, extracted lignin content, and yield of regenerated biomass to those obtained using [EMIM]Ac only. Mai *et al*. (2014) proposed a microwave-assisted method for the pretreatment of rice straw using the [EMIM]Ac/DMSO co-solvent system. The microwave-assisted pretreatment provided at least 22 times faster enzymatic saccharification than that of un-treated rice straw due to more efficient lignin extraction, lesser content of crystalline cellulose, and lower residual ILs in the treated rice straw. Han *et al*. (2017, 2020a) reported the co-solvent systems of [EMIM]Ac/DMF and DMAc for improved enzymatic saccharification of pussy willow. Used as co-solvents, both DMF and DMAc enable higher biomass loadings by lowering the viscosity and the cost of the ILs used in the pretreatment. Pretreatment with [EMIM]Ac and its mixtures with DMF and DMAc at a content of more than 50% showed the similar enzymatic saccharification efficiency to that obtained using pure [EMIM]Ac.

In this study, the co-solvent systems were prepared using different combinations of three ILs, namely [EMIM]Ac, [BMIM]Br, and [MMIM]$MeSO_4$ and three OSs, namely DMF, DMAc, and DMSO. Three ILs were selected because of their high solubility of all biomass components (Zhu *et al*. 2013; Cao *et al*. 2016). The effects of these co-solvent systems on the pretreatment for the enzymatic saccharification of pussy willow and Korean pine were investigated.

EXPERIMENTAL

Materials

The pussy willow (*Salix gracilistyla* Miq.) and Korean pine (*Pinus koraiensis* Siebold & Zucc.) samples were ground to a particle size of less than 40 mesh and extracted at 85 °C for 5 h with an ethanol/benzene mixed solution (1/2, v/v) followed by vacuum drying at 85 °C. [EMIM]Ac, [BMIM]Br, and [MMIM]$MeSO_4$ were purchased from IoLiTec (Heilbronn, Germany), and the other chemicals used in this study were purchased

from Daejung Chemicals & Metals (Siheung, Korea). Acremonium cellulase and Optimash BG were obtained from Meiji Seika Co. (Tokyo, Japan) and Genencor International, Inc. (Palo Alto, CA, USA), respectively.

Chemical composition analysis

The chemical composition of the raw materials was analyzed as follows. To measure the holocellulose content, the extracted sample (1 g) was reacted with the mixed solvent of $NaClO_2$ (0.4 g) and distilled water (60 mg) mixed with acetic acid (80 μL) in a water bath at 80 °C for 1 h, and this procedure was repeated five times by adding the same amount of $NaClO_2$ and acetic acid for complete removal of lignin. The delignified product was filtered, washed several times with distilled water, dried at 40 °C for 24 h, and weighed to determine the holocellulose content (Wise *et al*. 1946). The holocellulose sample (0.6 g) was further treated by a 17.5% sodium hydroxide solution (17.5 mL) and neutralized using a 10% acetic acid solution (28 mL). After washing with distilled water, the filtered product was vacuum-dried at 40 °C to obtain α-cellulose. The hemicellulose content was calculated by subtracting the α-cellulose content from the holocellulose content. The Klason lignin content was determined as the insoluble residue amount obtained by the treatment with a 72% sulfuric acid solution for 1 h at room temperature, diluting to 3%, and reacting in an autoclave (1 h, 120 °C).

Pretreatment

The mixture was prepared by mixing one of the three ILs ([EMIM]Ac, [BMIM]Br, and [MMIM]$MeSO_4$) and one of the three OSs (DMF, DMAc, and DMSO) at the weight ratios of 7/3, 5/5, and 3/7. Wood flour (pussy willow and Korean pine) was added to ILs, OSs, and their mixtures with a solid loading of 15 wt%. The mixtures were stirred with a vortex mixer and left to stand at 120 °C for 2 h in a dry oven. The pretreated products were precipitated in distilled water to separate the WSF and the solvents. The precipitated products were vacuum-filtered using PTFE membrane filters (ADVANTEC®, Toyo Roshi Kaisha, Ltd., Tokyo, Japan), and the obtained residues were freeze-dried using an FDB-5503 instrument (Operon Co., Ltd., Gimpo, Korea) and used for enzymatic saccharification and other characterizations.

Enzymatic saccharification and acid hydrolysis

Enzymatic saccharification was conducted in a shaking incubator (150 rpm) at 50 °C for 72 h. An enzyme cocktail of acremonium cellulase (15 FPU per gram of substrate) and Optimash BG (0.2%, v/v) was used as a supplement to β-xylosidase. The pretreated substrates (200 mg) were added to the enzyme cocktail at a solid loading of 2% in a sodium acetate buffer (50 mM, pH 5), and hydrolysates (1 mL) were aliquoted at 3, 6, 12, 24, 48, and 72 h. After heating at 95 °C for 15 min in a heating block, the enzyme activation was stopped, and the samples were stored at 4 °C before HPLC separation.

Acid hydrolysis was also conducted according to the NREL protocol (Sluiter *et al*. 2008) for the compositional measurement of the monosaccharides in the pretreated product. The pretreated products (100 mg) were added to a 72% sulfuric acid solution (0.7 mL), and the mixture was thoroughly mixed using a vortex mixer and incubated in a shaking incubator for 1 h at 250 rpm. The reactant was diluted to 4% by adding deionized water (19.4 mL) and was reacted again at 120 °C for 1 h in an autoclave. The hydrolysate was neutralized by adding a calcium carbonate solution.

For HPLC measurements, both enzymatic and acidic hydrolysates were centrifuged at 7000 rpm for 15 min and filtered using a syringe filter with a pore size of 0.2 μm (Sartorius, Goettingen, Germany).

Analysis

The viscosities of ILs, OSs, and their mixtures were measured at 25 °C using an LVDV-II+ Brookfield viscometer (Brookfield Engineering Laboratories, Inc., Middleboro, MA, USA) with a spindle number of 18 (shear rate range: 0 to 132.0/sec; viscosity range: 3 to 10,000 cP).

Morphology was observed using a scanning electron microscope (SEM, S-4800, Hitachi, Tokyo, Japan) at the Central Laboratory of Kangwon National University. The sample was freeze-dried and coated with platinum using a vacuum evaporator (JEE-400, JEOL Ltd., Tokyo, Japan) with an electric current of 20 mA.

The monosaccharides were analyzed with an HPLC system (YL9100; Young Lin Instrument Co., Ltd., Anyang, Korea) equipped with an Aminex HPX-87P column (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The mobile phase was deionized water, and the flow rate was 0.6 mL/min at 85 °C. The glucose and xylose yields in the enzymatic hydrolysate were calculated according to Eq. 1:

$$= \frac{\text{Mass of glucose (or xylose) in enzymatic hydrolysates (g)} \times 0.9(0.88)}{\text{Mass of glucan (or xylan) in pretreated wood (g) by acid hydrolysis}} \times 100 \quad (1)$$

RESULTS AND DISCUSSION

As shown in Table 1, according to the quantitative analyses, the α-cellulose, hemicellulose, Klason lignin, and extractives contents of the un-treated Korean pine were 35.6, 23.7, 38.5, and 6.3 wt%, respectively. Compared to pussy willow, the hemicellulose content and the lignin and extractives of Korean pine contents were lower and higher, respectively.

Table 1. Chemical Composition of the Un-treated Wood

Compounds	Pussy Willow (wt%)	Korean Pine (wt%)
α-cellulose	37.6	35.6
Hemicellulose	35.9	23.7
Klason lignin	25.0	38.5
Extractives	5.6	6.3

Figure 1 shows the viscosity of ILs and their mixtures with different ratios of ILs such as [EMIM]Ac, [MMIM]$MeSO_4$, and [BMIM]Br, and OSs such as DMF, DMAC, and DMSO. The use of the mixture of ILs and OSs in order to reduce the viscosity of ILs has been suggested in several studies, and it was shown that the addition of the OS does not significantly affect the specific interactions between cations and anions or between the IL and the lignocellulosic biomass (Andanson *et al*. 2014; Han *et al*. 2017; Wu *et al*. 2013). The viscosity of [BMIM]Br was more than 3000 cP, while the viscosities of the other ILs

([EMIM]Ac and [MMIM]$MeSO_4$) were lower than 150 cP. The viscosities of DMF, DMAC, and DMSO were 1.35, 1.53, and 2.64 cP, respectively. The addition of OSs to ILs dramatically decreased the viscosity of ILs and their mixtures, showing values lower than 100 cP, regardless of the IL type. The viscosity of the mixtures with DMF was lowest in all samples with different IL/OS ratios. In particular, when 30% DMF was added to [BMIM]Br, the viscosity drastically decreased by approximately 90 times from 3090 cP to 34.7 cP and was 51.8 and 98.6 cP in mixtures with 30% DMAc and DMSO, respectively. This low viscosity was helpful for handling at a relatively high biomass loading of 15% in ILs. Wu *et al*. (2013) also reported the effect of DMSO addition on the viscosity of [EMIM]Ac, showing that an exponential decrease in the viscosity is obtained by increasing the DMSO ratio in the mixtures. Yang *et al*. (2017) used four organic solvents, i.e., DMF, DMAc, DMSO, and pyridine (PYR) for lowering the viscosity of [BMIM]Cl. DMF showed the most considerable effect to lower the viscosity of pure IL, which is similar with our results. They have also studied the microstructure of the mixtures using FTIR, aiming to understand further the relationship between the properties and the structures of the mixtures. They reported that C4-H in the IL and PYR may form the weakest hydrogen-bonding but other solvents did not show significant hydrogen-bonding formations.

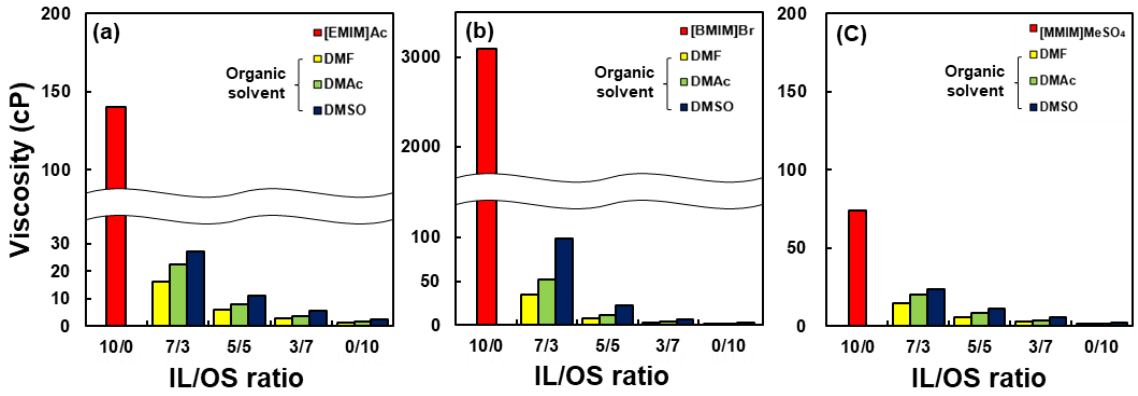

Fig. 1. Effect of ILs/OSs ratio on the viscosity of the mixtures

Figure 2 shows the obtained WSFs of the pussy willow and Korean pine pretreated with ILs, OSs, and their mixtures with different mixing ratios. Most of the components in WSF would be the small molecular products from hemicellulose and lignin. Silva *et al*. (2011) reported that a cellulose-rich fraction could be recovered as a consequence of the loss of water-soluble lignin and hemicellulose during precipitation of the dissolved product. For pussy willow, the WSF of the products obtained using pretreatments with pure ILs, *i.e*., [EMIM]Ac, [BMIM]Br, and [MMIM]$MeSO_4$ were 23.0, 23.1 and 23.5%, respectively.

By contrast, the WSFs for Korean pine were 9.7, 12.7, and 13.0, approximately twice smaller than the corresponding values for pussy willow. For both species, the values obtained using pretreatment with pure ILs showed a decreasing trend with increasing OS in the mixtures, but the amount of the decrease was small. This result indicates that hardwood (pussy willow) is more susceptible to be dissolved in ILs than softwood (Korean pine).

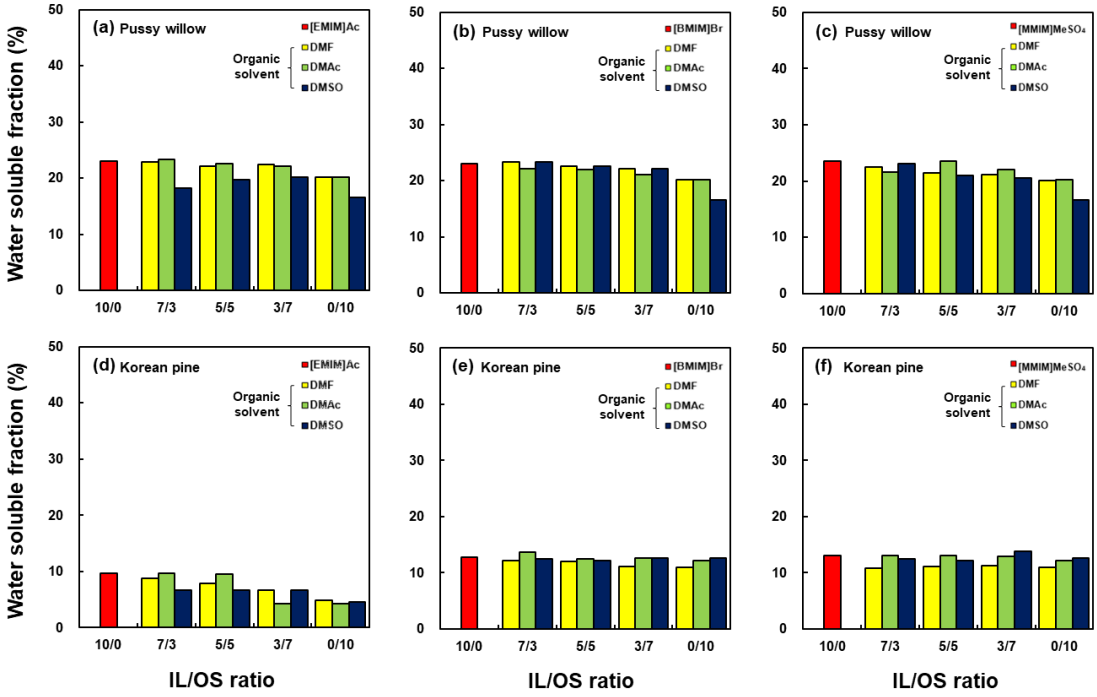

Fig. 2. Effect of the ILs/OSs ratio on the WSF of pussy willow and Korean pine

The surface morphology of the un-treated and pretreated products by ILs, OSs, and their mixtures of pussy willow (Fig. 3) and Korean pine (Fig. 4) was observed by SEM. Un-treated samples of both species exhibited a highly ordered fibrillar and compact morphology. In contrast, all pretreated products significantly alter the fibrillar structure and the surfaces became rougher after pretreatment due to the disruption of the cell wall structure (Weerachanchai and Lee 2013). Some lignin particles extracted from the cell wall were also observed in Korean pine pretreated with [MMIM]$MeSO_4$ (Fig. 4g). IL can cause the cleavage of the chemical bonds between lignin and hemicellulose and the disruption of hydrogen bonding in the cellulosic network, resulting in the increase of the porosity and the surface area (Alayoubi *et al*. 2020). It will promote an increase in enzymatic efficiency (Dong *et al*. 2019). Among the ILs, the pretreatment with [EMIM]Ac significantly increased the porosity and surface area than other ILs used. Hu *et al*. (2018) also reported that smooth and compact morphology of un-treated sample limited cellulase contact sites, lowering enzymatic hydrolysis efficiency, whereas pretreatment using [BMIM]BF_4 made the loose porous structure, resulting in the increase in enzymatic hydrolysis efficiency.

Figures 5, 6 and 7, 8 show the effect of the IL/OS ratio on the yield of glucose and xylose obtained by enzymatic saccharification from the pretreated pussy willow and Korean pine, respectively. The yields of glucose and xylose were calculated based on the mass of glucan and xylan in the pretreated product obtained by acid hydrolysis. The yields of glucose and xylose were 14.34 and 8.03% in un-treated pussy willow and 8.53 and 7.86% in un-treated Korean pine, respectively, for 72 h saccharification. In all samples, both yields increased with increasing saccharification time, showing a rapidly increasing trend up to the saccharification time of 12 h and followed by a gradual increase.

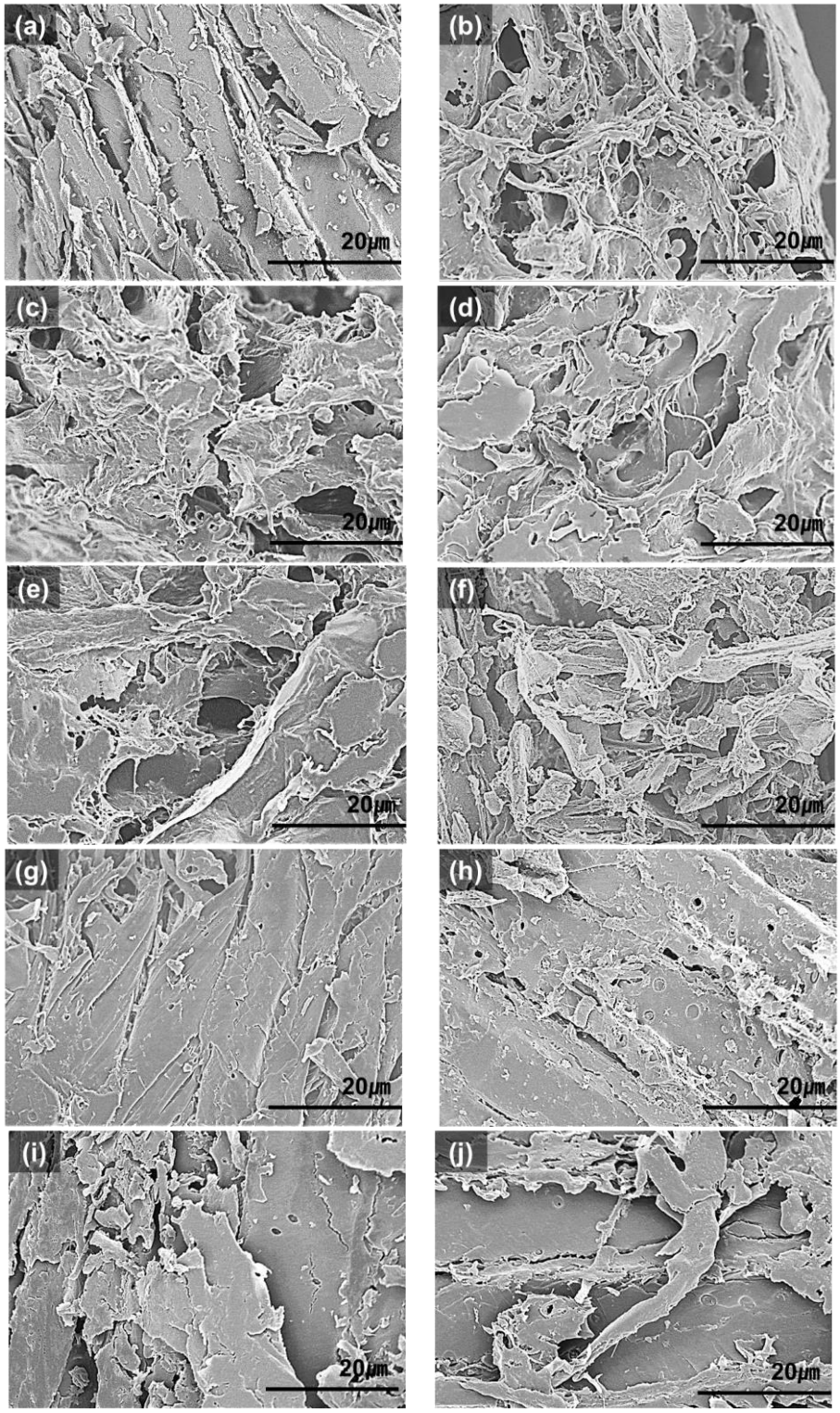

Fig. 3. Morphology of un-treated product (a) and pretreated products by [EMIM]Ac (b), its mixtures with 50% OSs (DMF (c); DMAc (d); DMSO (e)), [BMIM]Br (f), [MMIM]$MeSO_4$ (g) and OSs (DMF (h); DMAc (i); DMSO (j)) of pussy willow

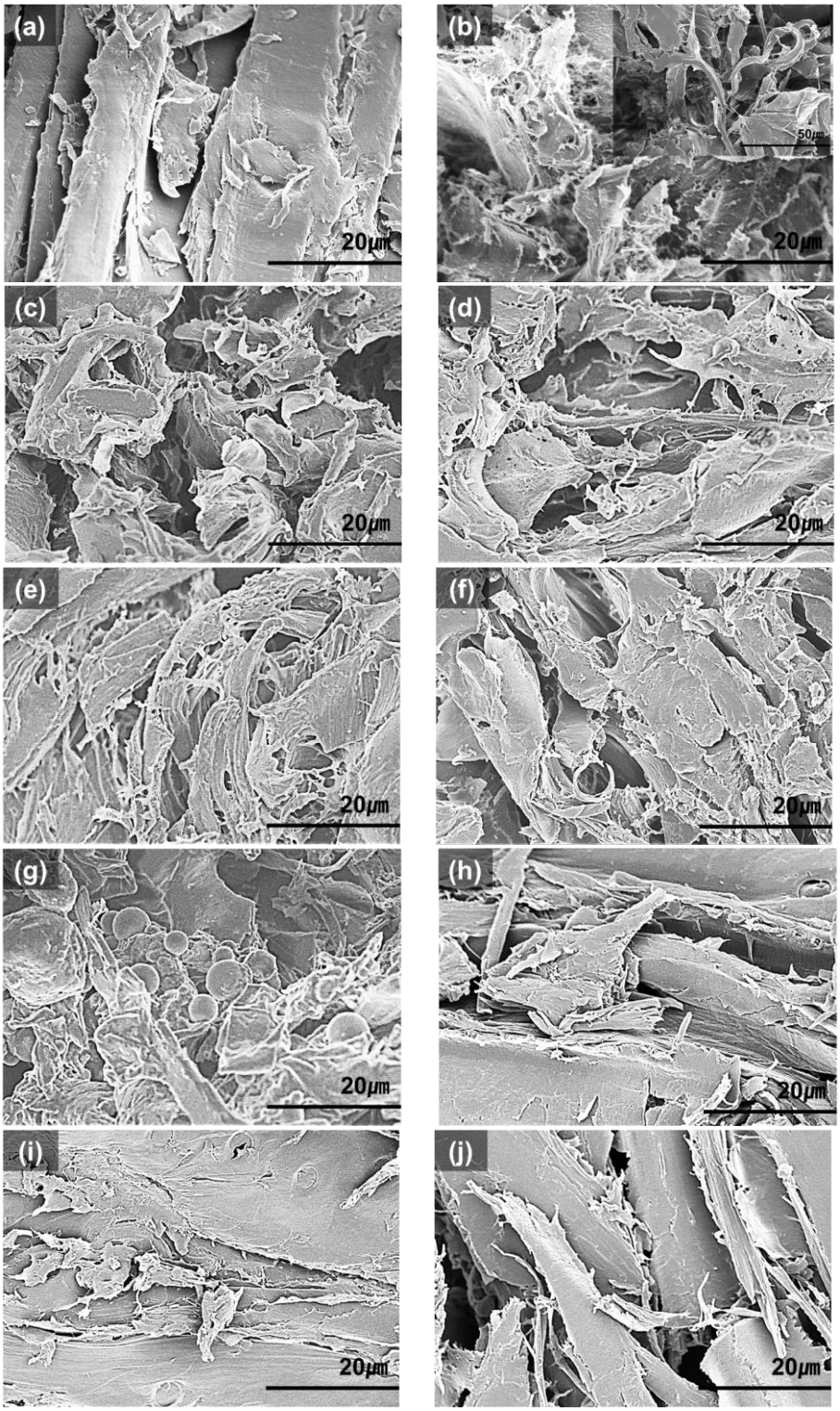

Fig. 4. Morphology of un-treated product (a) and pretreated products by [EMIM]Ac (b), its mixtures with 50% OSs (DMF (c); DMAc (d); DMSO (e)), [BMIM]Br (f), [MMIM]$MeSO_4$ (g), and OSs (DMF (h); DMAc (i); DMSO (j)) of Korean pine

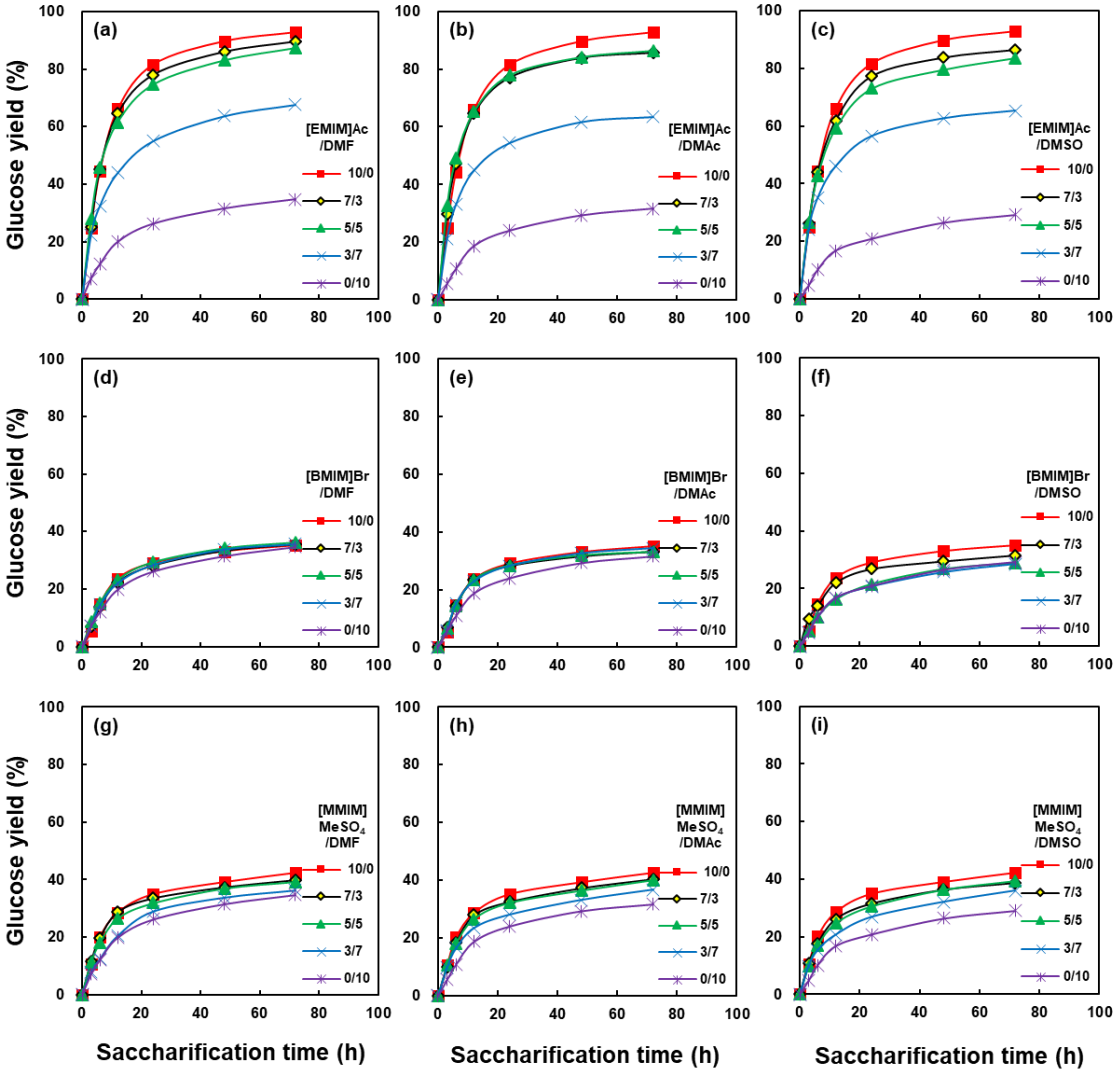

Fig. 5. Effect of the IL/OS ratio on the glucose yield of enzymatic saccharification for pretreated pussy willow

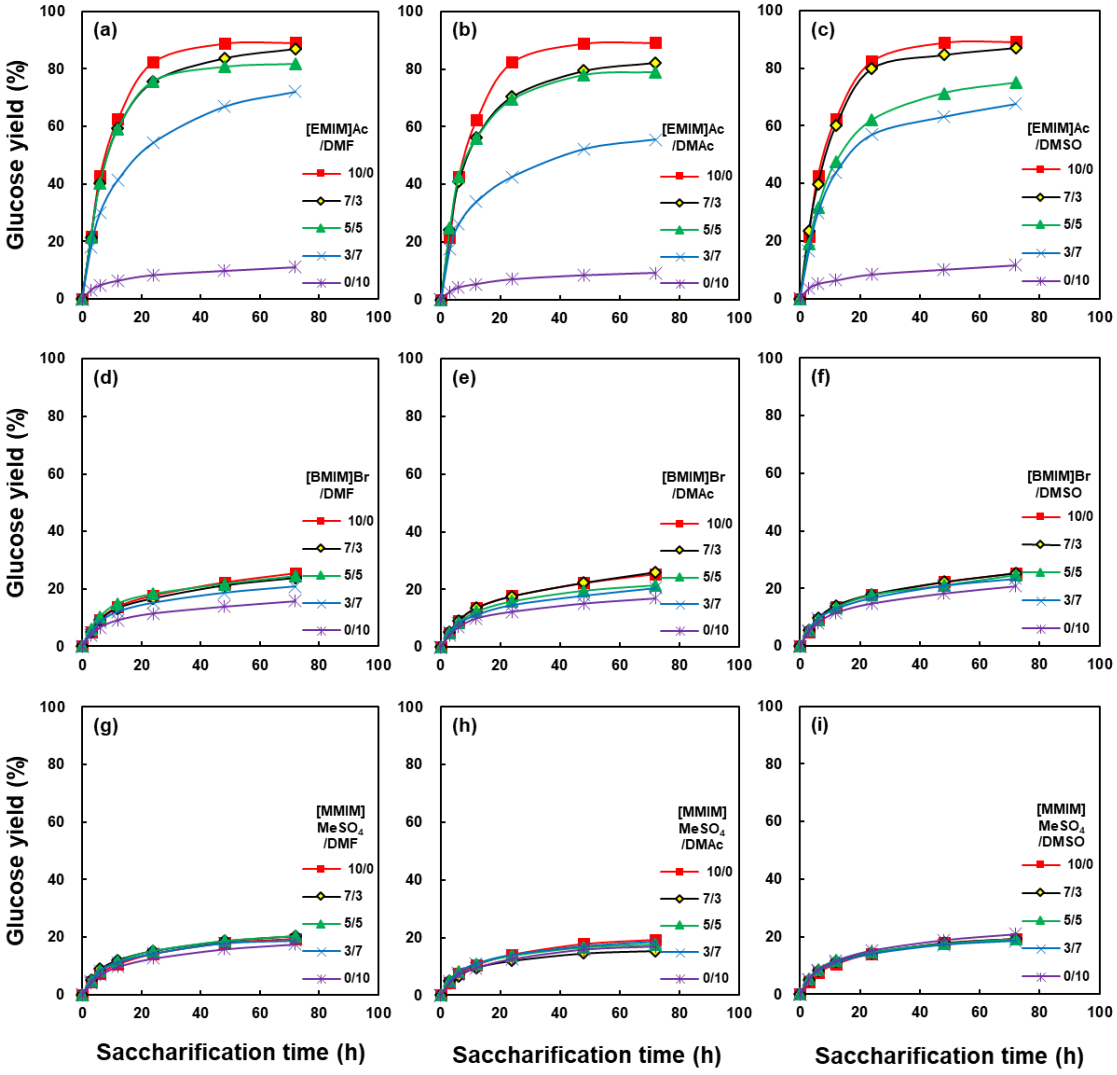

Fig. 6. Effect of the IL/OS ratio on the glucose yield of enzymatic saccharification for pretreated Korean pine

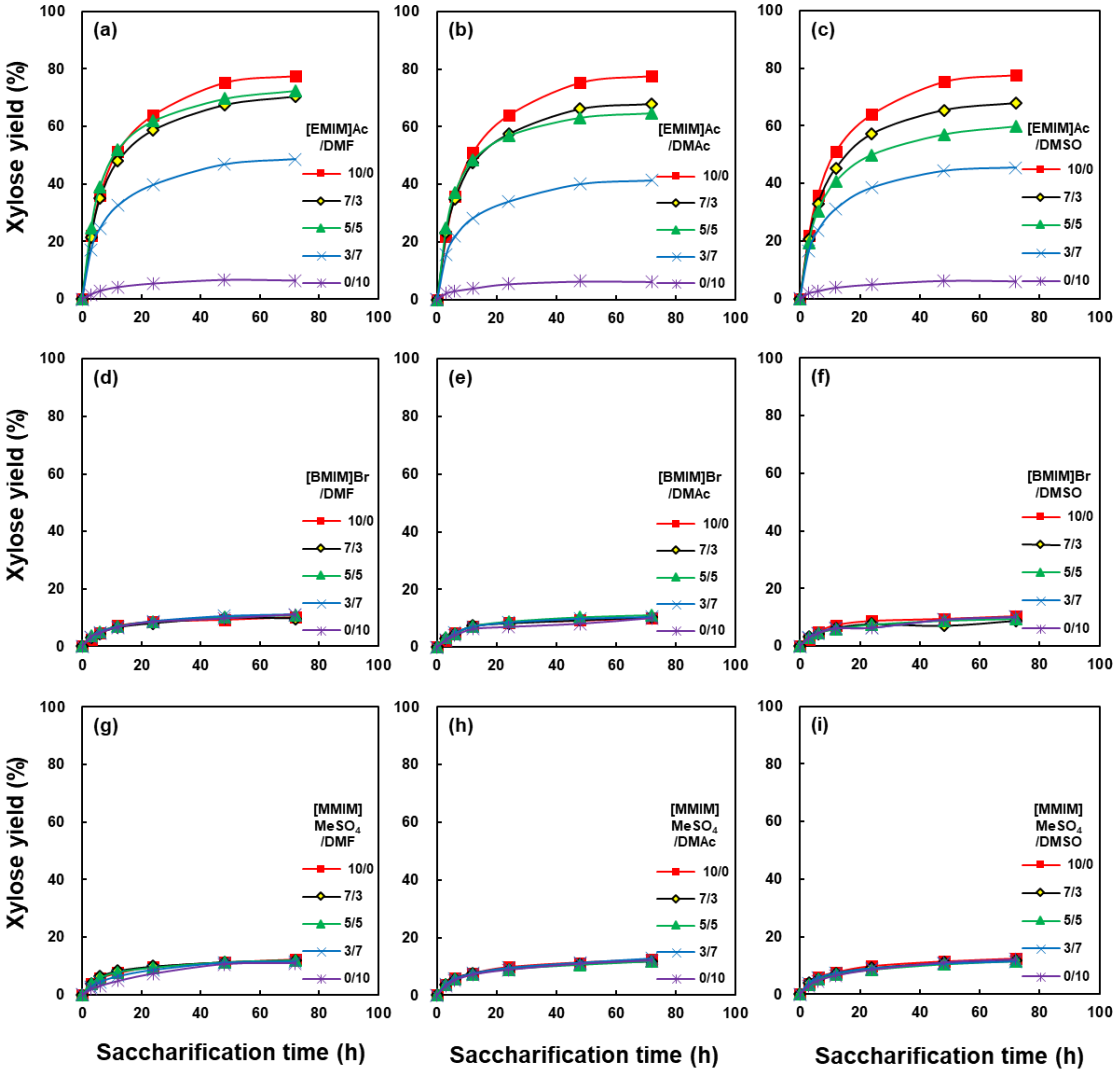

Fig. 7. Effect of the IL/OS ratio on the xylose yield of enzymatic saccharification for pretreated pussy willow

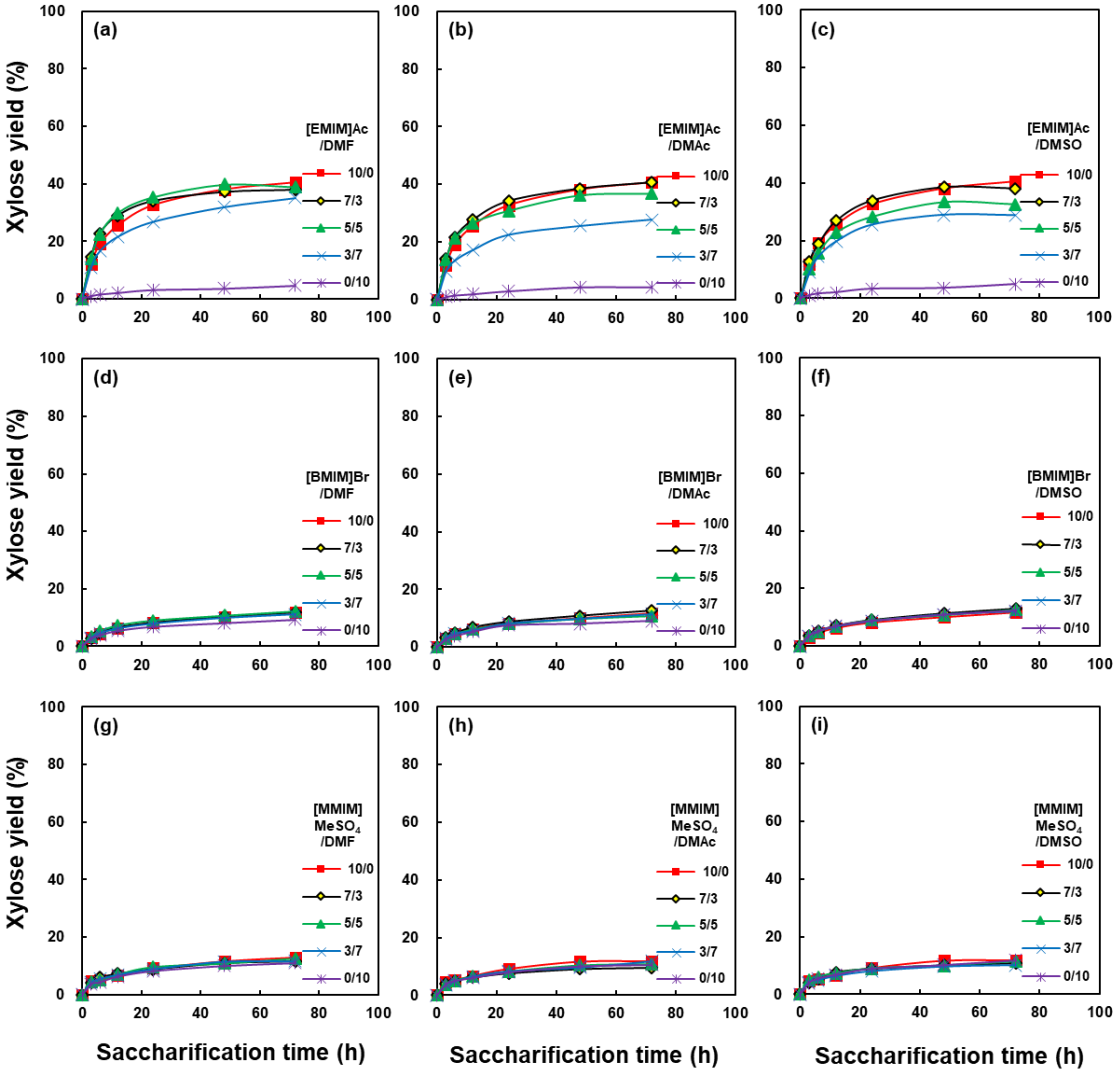

Fig. 8. Effect of the IL/OS ratio on the xylose yield of enzymatic saccharification for pretreated Korean pine

In both species, all saccharification yields were higher in [EMIM]Ac and its mixtures than in [BMIM]Br, [MMIM]$MeSO_4$, and their mixtures. However, there was no significant difference for both saccharification yields from those obtained for the products pretreated by [BMIM]Br, [MMIM]$MeSO_4$, and their mixtures. Based on the saccharification time of 72 h, for pussy willow, the glucose yields were in the range of 28.5 to 35.1% and 36.0 to 42.4% in [BMIM]Br and its mixtures and [MMIM]$MeSO_4$ and its mixtures, respectively, while for Korean pine, the corresponding glucose yields were in the range of 20.5 to 25.5% and 15.4 to 19.3%, respectively. Xylose yields in both ILs ([BMIM]Br and [MMIM]$MeSO_4$) and their mixtures were less than 10% for both species, showing slightly larger values in hardwood than in softwood. For both species, these saccharification yields were not significantly different from those obtained from the

product pretreated by the pure OS, showing no effect of ILs pretreatment on the improvement of enzymatic saccharification.

For both species, differences in the yields of glucose and xylose were obtained for the products pretreated by [EMIM]Ac and its mixtures. For the mixture with 50% or less of OS for both species, the glucose yield was found to be greater than 70% for the saccharification time of 72 h, regardless of the OS type. The mixture with 70% OS amount showed greater than 50% glucose yield for both species, and are still higher than those obtained using the pure OS. The glucose yields for pussy willow were higher by approximately 34.7, 31.6, and 29.1% for DMF, DMAc, and DMSO, respectively, while increases of 17.5, 17.0, and 19.3% were obtained for Korean pine. While Korean pine and pussy willow showed similar trends, the yields for Korean pine were slightly smaller than those for pussy willow. The dependence of the xylose yield on the IL/OS ratio was also similar to that for the glucose yield for both species. However, the overall values obtained after saccharification for 72 h were higher for pussy willow than for Korean pine because xylan is the main hemicellulose component in hardwood.

CONCLUSIONS

1. Pretreatments using three different ionic liquids (ILs), namely [EMIM]Ac, [BMIM]Br, and [MMIM] $MeSO_4$ and their mixtures obtained by combinations with three different organic solvents (OSs), namely dimethyl formamide (DMF), *N*,*N*-dimethylacetimide (DMAc), and dimethyl sulfoxide (DMSO), were conducted to improve the enzymatic saccharification of pussy willow and Korean pine.
2. The viscosity was reduced by increasing the OSs amount in the mixtures, regardless of the solvent type. In particular, the viscosity of [BMIM]Br was drastically decreased by OS addition.
3. For both species, water-soluble fractions (WSFs) obtained using pure ILs decreased with increasing OS amount in the mixtures, and the WSFs for pussy willow were twice as large as those of Korean pine.
4. For both species, higher yields of glucose and xylose were obtained using [EMIM]Ac and its mixtures than using [BMIM]Br, [MMIM]$MeSO_4$, and their mixtures. However, for both glucose and xylose, the differences between the products obtained using pretreatment by [BMIM]Br, [MMIM]$MeSO_4$, and their mixtures were small.

ACKNOWLEDGMENTS

This study was funded by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (Grant No. 2018R1A6A1A03025582).

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Article submitted: March 31, 2020; Peer review completed: June 20, 2020; Revised version received and accepted: November 13, 2020; Published: November 24, 2020.
DOI: 10.15376/biores.16.1.455-469