Pretreatment with [EMIM]Ac/DMAc Co-solvent to Improve Enzymatic Saccharification of Pussy Willow (*Salix gracilistyla* Miq.)

Song-Yi Han,[a] Chan-Woo Park,[a] Fauzi Febrianto,[b] Nam-Hun Kim,[a] and Seung-Hwan Lee [a,]*

Pretreatment of *Salix gracilistyla* Miq. with 1-ethyl-3-methylimidazolium acetate ([EMIM]Ac) together with N,N-dimethylacetamide (DMAc) as a co-solvent achieved an improvement in enzymatic saccharification yield in comparison to the raw material without pretreatment. The effects of the [EMIM]Ac/DMAc ratio (7:3, 5:5, and 3:7), pretreatment temperature (80 to 140°C), and pretreatment time (1 to 3 h) on enzyme saccharification were investigated. Acremonium cellulase and Optimash BG were used as the enzyme cocktail. With addition of DMAc to [EMIM]Ac, the viscosity of the co-solvent was drastically decreased. With increasing pretreatment temperature and time, the water soluble fraction increased at all [EMIM]Ac/DMAc ratios. The cellulose crystallinity also increased with increasing amount of DMAc in the co-solvent but decreased with increasing pretreatment temperature. The yields of glucose and xylose increased with increasing [EMIM]Ac amounts in the co-solvent, varying from 72.8% to 84.3% and 75.3% to 88.1%, respectively, for all [EMIM]Ac/DMAc ratios. These values were markedly higher than the glucose and xylose yields of 25.7% and 8.4%, respectively, obtained with DMAc alone and comparable to the 86.7% and 83.9% yields, respectively, obtained with [EMIM]Ac alone. The highest glucose and xylose yields obtained under the investigated pretreatment conditions were 84.3% and 88.1%, respectively.

Keywords: Pretreatment; Ionic liquid; 1-Ethyl-3-methylimidazolium acetate ([EMIM]Ac); N,N-Dimethylacetamide (DMAc); Enzymatic saccharification

*Contact information: a: Department of Forest Biomaterials Engineering, College of Forest and Environmental Sciences, Kangwon National University, Chuncheon 24341, Republic of Korea; b: Department of Forest Products, Faculty of Forestry, Bogor Agricultural University, Bogor 16680, Indonesia; *Corresponding author: lshyhk@kangwon.ac.kr*

INTRODUCTION

Lignocellulosic biomass is the most abundant solid renewable resource on earth. It has attracted attention as an alternative energy source due to its renewability and sustainability. It is mainly composed of cellulose and hemicellulose, which are polysaccharides, and lignin, which is an aromatic compound. Woody biomass is expected to replace the first generation feedstocks, such as corn and sugar cane, for bioenergy production (Kang *et al.* 2014; Zabed *et al*. 2016; Manochio *et al*. 2017). However, compared to the first generation feedstocks, it is more difficult to convert lignocellulosic biomass into bioenergy through chemical and biological treatment without a pretreatment process. The conversion efficiency of lignocellulosic biomass largely depends on the pretreatment process (Hendriks and Zeeman 2009; Ding *et al.* 2012). The primary purpose of pretreatment is to improve the biodegradability of polysaccharide components through the effect of delignification or lowering the degree of polymerization (DP) and cellulose crystallinity (Brodeur *et al*. 2011; Willberg-Keyriläinen *et al*. 2019).

Recently, pretreatments using ionic liquids (ILs) have been introduced as a more environmentally friendly approach than conventional chemical pretreatments (Isik *et al*. 2014; Ungurean *et al*. 2014; Cvjetko Bubalo *et al*. 2015; Wang *et al*. 2017). Compared to conventional volatile organic solvents, ILs have many merits, such as high thermal stability, non-volatility, non-flammability, low melting points, and excellent solubility (Earle and Seddon 2000; Shamsuri and Abdullah 2011; Das and Roy 2012). They can dissolve chemical compounds that are difficult to solubilize in conventional solvents (Luo *et al*. 2013), particularly for lignocellulosic biomass (Hossain and Aldous 2012). However, ILs also have some limitations, including high cost and low solid loading due to their high viscosity (Marsh *et al*. 2004; Hou *et al*. 2017). For example, the viscosity of ILs, which are known to have a positive effect on the pretreatment of lignocellulosic biomass, is generally 1- to 3-fold higher than that of conventional organic solvents, with values ranging from 4.8 to 1110 mPa·s depending on the cation and anion (Zhang *et al*. 2006).

The high viscosity of ILs will negatively affect the cost and pretreatment capability of the pretreatment process, thus requiring a high temperature to achieve a good pretreatment effect (Reddy 2011; Weerachanchai and Lee 2013). This high reaction temperature may cause undesired side reactions, for example, the production of fermentation inhibitors such as furfural and hydroxymethylfurfural from polysaccharides at temperatures greater than 150 °C (Weerachanchai *et al*. 2012). In this context, several approaches have been proposed to decrease the viscosity of ILs through introduction of organic solvents for improvement of their pretreatment effect of lignocellulosic biomass (Tokuda *et al*. 2005; Khupse and Kumar 2010). Xu *et al*. (2013) reported that a co-solvent of 1-butyl-3-methylimidazolium acetate ([Bmim]Ac) and dimethyl sulfoxide (DMSO) showed a higher solubility of cellulose than [Bmim]Ac alone, even at room temperature (25 °C). Tao *et al*. (2016) also reported that the addition of DMSO to 1-allyl-3-methylimidazolium chloride ([Amim]Cl) improved the dissolution of cellulose 12 to 15% at 30 °C. The thermal stability of cellulose regenerated from [Amim]Cl/DMSO was also significantly higher than that with pure [Amim]Cl.

The author group has previously studied pretreatments using IL co-solvent systems with organic solvents, such as DMF, DMAc, and DMSO, that are known to have a good affinity to the cellulose in lignocellulosic biomass. Wu *et al*. (2013) reported that a co-solvent of [EMIM]Ac and DMSO was effective for improving the saccharification yield of eucalyptus wood. The glucose yield of a sample pretreated with this co-solvent ([EMIM]Ac/DMSO ratio = 2:3) at 140 °C reached approximately 95% after enzymatic hydrolysis for 72 h. Han *et al*. (2017) also reported the pretreatment effect of an [EMIM]Ac/DMF co-solvent system on the enzymatic saccharification of pussy willow. This system effectively lowered the viscosity and resulted in the increased solid loading in the co-solvent. The crystallinity of the pretreated product could be reduced by increasing the amount of [EMIM]Ac in the co-solvent. The glucose yield of the sample pretreated with a co-solvent with less than 70% DMF content was comparable to that pretreated with [EMIM]Ac alone. In this study, DMAc rather than DMSO or DMF, was used as the counterpart organic solvent with [EMIM]Ac. The effect of the resulting co-solvent on pretreatment efficiency to improve the enzymatic saccharification of pussy willow was investigated and discussed in comparison to previous research that used DMSO and DMF. Pussy willow is known as the promising feedstock for biorefinery process including bioenergy production because it is fast growing with excellent sprouting and has a better cultivation yield per unit area than another short rotation forestry such as poplar and redwood (Aylott *et al.* 2008; Serapiglia *et al.* 2014; Stolarski *et al.* 2017). In particular, pussy willow is abundantly growing throughout the riparian area in the Republic of Korea (Chun *et al.* 1999; Lee *et al.* 2018).

EXPERIMENTAL

Materials

Pussy willow (*Salix gracilistyla* Miq.) was provided by the Department of Plant Genetic Engineering of Forest Resources at Kangwon National University (Chuncheon, Korea). The provided material was ground to a size of 200 μm and vacuum-dried at 40 °C for more than 24 h. The [EMIM]Ac and DMAc were purchased from IoLiTec-Ionic Liquids Technologies GmbH (Heilbronn, Germany) and Daejung Chemicals & Metals Co., Ltd. (Siheung, Korea), respectively. Acremonium cellulase (Meiji Seika Co., Tokyo, Japan) derived from the fungus *Acremonium cellulolyticus* with the specific activity of endoglucanase, xylanase, and mannanase (Fujii *et al*. 2009), and Optimash BG (Genencor International, Inc., Palo Alto, CA, USA) with β-xylosidase activity were used for the enzyme cocktail.

Methods

Pretreatment

The co-solvent was prepared from mixing [EMIM]Ac and DMAc at weight ratios of 7:3, 5:5, and 3:7. The [EMIM]Ac, DMAc, and the co-solvents were added to wood powder to form a solid content of 15 wt%. The mixtures were stirred with a vortex mixer and reacted at 80, 100, 120, and 140 °C for 1 to 3 h in an oven. The pretreated product was then precipitated in distilled water to separate the water soluble fraction and solvents. The precipitated product was vacuum-filtered by PTFE membrane filters (ADVANTEC®, Toyo Roshi Kaisha, Ltd., Tokyo, Japan) with a pore size of 0.2 μm. The filtered product was oven-dried at 100 °C and used for enzymatic saccharification and other characterizations.

Acid and enzymatic hydrolysis

Acid hydrolysis for compositional analysis was conducted according to the Laboratory Analytical Procedure written by the US National Renewable Energy Laboratory (Sluiter *et al*. 2008). The pretreated products (100 mg) were added to 72% sulfuric acid (1 mL). After thoroughly mixing in a vortex mixer, the mixture was incubated in a shaking incubator (VS-101si; Vision Scientific, Co., Ltd., Daejeon, Korea) at 30 °C for 1 h. The reacted product was then diluted to a concentration of 4% through the addition of deionized water, and heated at 120 °C for 1 h in an autoclave. The product was thus divided into acid-insoluble lignin and acid-soluble hydrolysate. The acid-soluble hydrolysate was neutralized by adding calcium carbonate for high-performance liquid chromatography (HPLC) analysis.

Enzymatic hydrolysis was conducted in shaking incubator at 50 °C for 72 h and employing an enzyme cocktail of Acremonium cellulase (15 FPU per gram of substrate) and Optimash BG (0.2%, v/v) as a supplement to β-xylosidase. The pretreated substrates (250 mg) were added to the enzyme cocktail at a solid loading of 2.5% in sodium acetate buffer (50 mM, pH 5). Hydrolysates (1 mL) were sampled at 3, 6, 12, 24, 48, and 72 h intervals. The enzyme activation was stopped by heating at 95 °C for 15 min in a heating block (HB-48-Set, Wisd; Daihan Scientific Co., Ltd., Wonju, Korea), and the samples were stored at 4 °C before HPLC separation.

Enzymatic and acidic hydrolytes were centrifuged at 7000 rpm for 15 min and filtered through a syringe filter of pore size 0.2 µm (Sartorius, Goettingen, Germany).

Measurements

The viscosities of the [EMIM]Ac, DMAc, and co-solvents were measured at 25 °C with 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/s; viscosity range: 3 to 10,000 cP). For X-ray diffraction (XRD) measurements, the disk pellet technique (1-cm diameter, 0.8-mm thickness) was applied. An RINT-TTR III diffractometer (Rigaku Corp., Tokyo, Japan) with Cu Kα radiation (λ = 0.1542 nm, generated at 50 kV and 300 mA) was used. The diffraction intensity was measured in the range of 2° to 60° at intervals of 0.02° and a scanning rate of 2°/min. Analysis of the monosaccharides was conducted 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 by following equation,

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

where the numbers 0.9 and 0.88 are conversion factors for glucose and xylose to glucan and xylan, respectively.

RESULTS AND DISCUSSION

Viscosity

DMAc is known as the counterpart solvent of lithium chloride (LiCl) for cellulose dissolution, and it also has good miscibility with [EMIM]Ac. Figure 1 shows the effect of the addition of DMAc to [EMIM]Ac on the viscosity of the co-solvent.

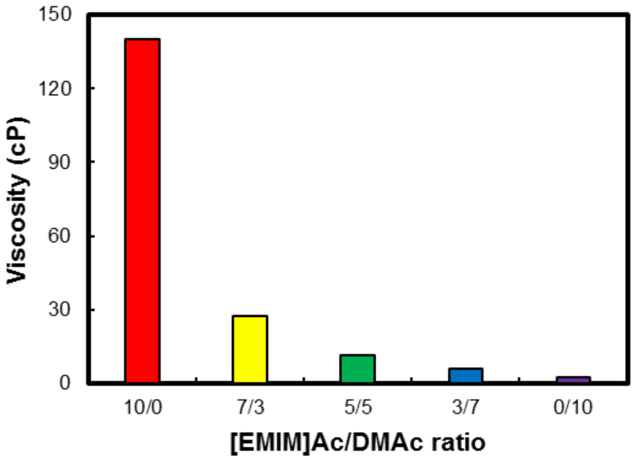

Fig. 1. Change in viscosity with the addition of DMAc to [EMIM]Ac

The DMAc with low viscosity (1.5 cP) drastically decreased the viscosity of co-solvent, which is beneficial for increasing the biomass loading in the co-solvent during pretreatment. The viscosity of the co-solvent decreased from 22.6 to 3.7 cP as a result of the increase of DMAc from 30 to 70 wt%. As described in the authors' previous studies (Wu *et al*. 2013; Han *et al*. 2017), the decrease in viscosity with the addition of DMF or DMSO to [EMIM]Ac led to improvement of the mixing efficiency at high biomass loading.

Water Soluble Fraction

Figure 2 shows the effects of the [EMIM]Ac/DMAc ratio, pretreatment time, and temperature on the amount of water soluble fraction obtained. The yields of water soluble fraction were 25.7, 26.8, and 21.3% at [EMIM]Ac/DMAc ratios of 7:3, 5:5, and 3:7, respectively. These values were slightly lower than the 28.8% obtained using [EMIM]Ac alone (Fig. 2a). Compared to the authors' previous study using an [EMIM]Ac/DMF co-solvent (Han *et al*. 2017), similar results were obtained with DMAc, but the amount of water soluble fraction (20.2%) obtained using only DMAc was higher than the 12% obtained using only DMF. There was no significant effect of the pretreatment time on the amount of water soluble fraction at 120 °C, with yields of 20.9 to 26.9% for all [EMIM]Ac/DMAc ratios (Fig. 2b). In contrast, an increasing trend in the water soluble fraction was observed for [EMIM]Ac/DMAc ratios of 7:3 and 5:5; however, there was no significant difference at temperatures from 80 to 120 °C for an [EMIM]Ac/DMAc ratio of 3:7 (Fig. 2c). The amount of water soluble fraction obtained at 140 °C was greater than 30% for all [EMIM]Ac/DMAc ratios.

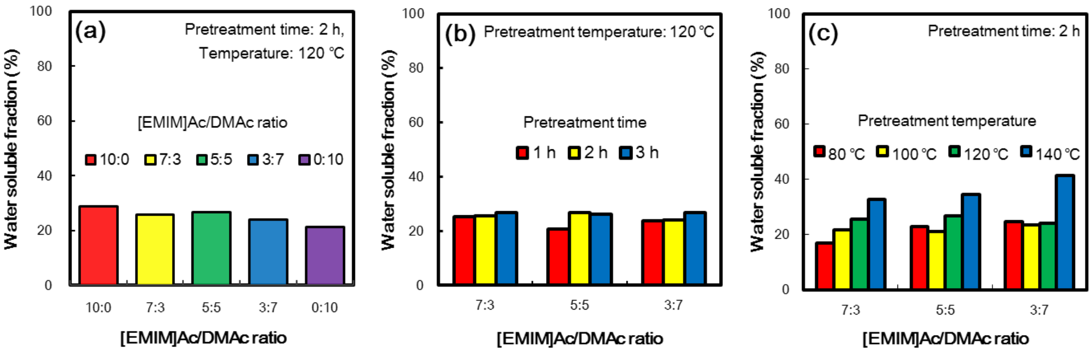

Fig. 2. Effect of the (a) [EMIM]Ac/DMAc ratio, (b) pretreatment time, and (c) temperature on the amount of water soluble fraction

Crystallinity

The cellulose crystallinity is known to be a major factor for improving enzymatic saccharification. The enzymatic saccharification of amorphous cellulose is much faster than that of crystalline cellulose because it is difficult for the enzyme to access the crystalline domain due to the strong hydrogen bonding between cellulose molecules. Additionally, it is commonly known that enzymes always hydrolyze the amorphous domain of cellulose first, which results in an increased crystallinity index (Ooshima *et al*. 1983; Zhang and Lynd 2004; Hall *et al*. 2010; Teixeira *et al*. 2015).

Figure 3 shows the effects of the [EMIM]Ac/DMAc ratio and pretreatment temperature on XRD pattern of the pretreated product. The XRD pattern of the DMAc pretreated product exhibited a typical cellulose I polymorph with major peaks from the

(101), (002), and (040) lattice planes, and appearing at 2*θ* = 15°, 22°, and 35°, respectively (Isogai *et al*. 1989; Park *et al*. 2010). However, the diffraction pattern corresponding to the cellulose I polymorph was diminished with increasing amount of [EMIM]Ac in the co-solvent and the increment of pretreatment temperature, while exhibiting a nearly amorphous pattern when the [EMIM]Ac amounts were over 50% and temperatures were higher than 120 °C. This change reflected a substantial disruption of the original crystal structure of the cellulose due to the breakdown of the hydrogen bonding network under the ionic liquid (Wu *et al*. 2013). In agreement with the enzymatic saccharification results described in the following section, the decrease in cellulose crystallinity and change in the crystalline structure to an amorphous pattern indicated improved enzymatic saccharification. Socha *et al*. (2013) reported that cellulose was less crystalline after pretreatment with [EMIM]Ac compared to untreated control samples.

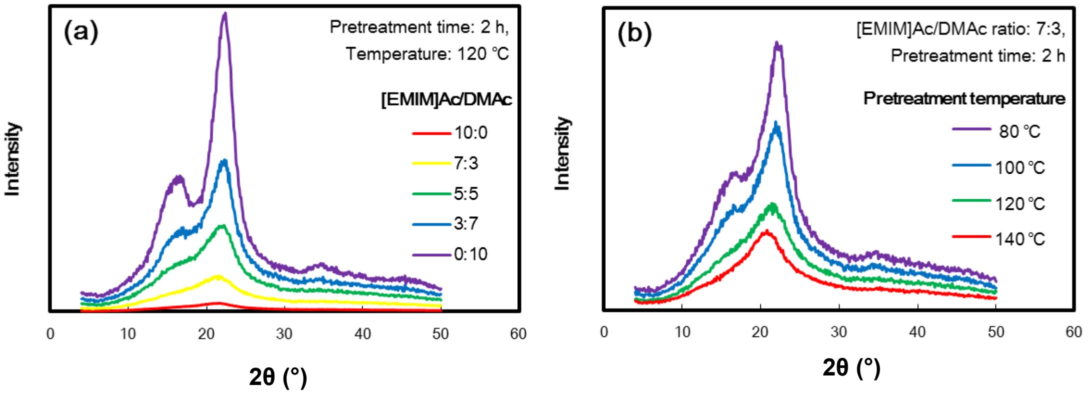

Fig. 3. X-ray diffraction patterns of the pretreated product with varying (a) [EMIM]Ac/DMAc ratios and (b) pretreatment temperatures

Enzymatic Saccharification Yield

Figure 4 shows the effect of the [EMIM]Ac/DMAc ratio on the yield of glucose and xylose at varying saccharification times. The yields of glucose and xylose were calculated based on the mass of glucan and xylan in the pretreated product obtained through acid hydrolysis. By extending the saccharification time, the yield of glucose and xylose increased, and there was a similar increasing trend shown in all samples. Compared to the saccharification yield obtained from the DMAc-pretreated product, the saccharification yields obtained from only [EMIM]Ac and its co-solvents with DMAc were markedly greater. A glucose yield of 72.8% and xylose yield of 75.3% were obtained through saccharification with an [EMIM]Ac/DMAc ratio of 3:7 for 72 h; these yields increased with further increase of the amount of [EMIM]Ac in the co-solvent. However, the xylose yield at [EMIM]Ac/DMAc ratio of 7:3 was the highest.

Figure 5 shows the effect of the varying pretreatment time on the enzymatic saccharification at different [EMIM]Ac/DMAc ratios. By lengthening saccharification time, the yields of glucose and xylose increased at all [EMIM]Ac/DMAc ratios. In addition, the yields of glucose and xylose also increased with increasing amounts of [EMIM]Ac in the co-solvent. When the saccharification yields were compared at a saccharification time of 72 h, the yields of glucose and xylose obtained with 2 h of pretreatment time were greater than those obtained with 3 h of pretreatment time at all [EMIM]Ac/DMAc ratios.

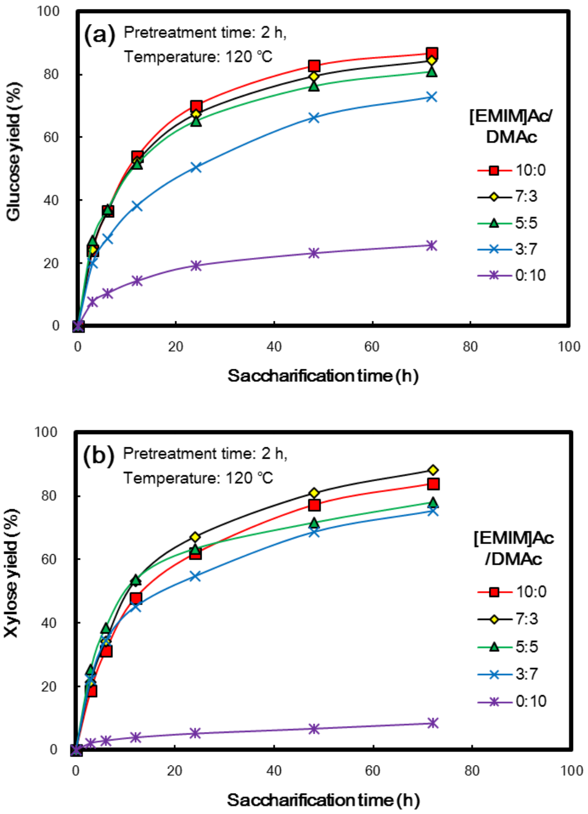

Fig. 4. Effect of the [EMIM]Ac/DMAc ratio on the yields of (a) glucose and (b) xylose with varying saccharification times (pretreatment conditions: 120 °C for 2 h)

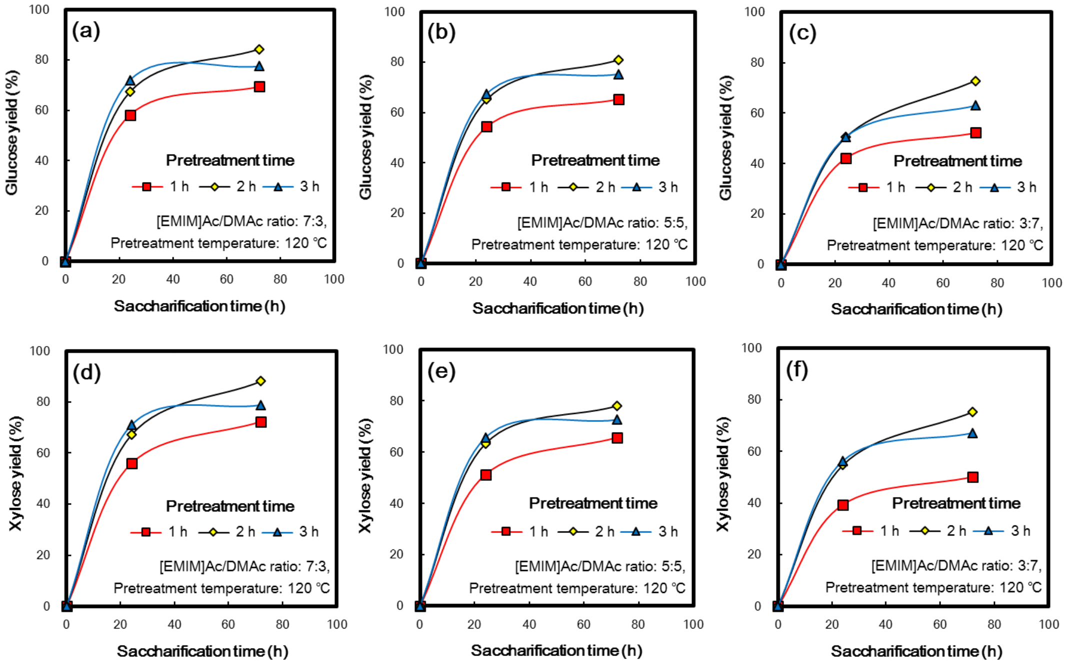

Fig. 5. Effect of pretreatment time on the enzymatic saccharification at [EMIM]Ac/DMAc ratio 7:3 (a, d), 5:5 (b, e), and 3:7 (c, f)

Figure 6 shows the effect of the pretreatment temperature on the enzymatic saccharification at different [EMIM]Ac/DMAc ratios. The yields of glucose and xylose increased with increment of [EMIM]Ac in the co-solvent. With increasing pretreatment temperature, the yields of glucose and xylose increased at all [EMIM]Ac/DMAc ratios. However, there was no significant difference in the glucose yield under pretreatment temperatures at 120 and 140 °C for 72 h: the glucose yield was approximately 80% at [EMIM]Ac/DMAc ratios of 7:3 and 5:5, and approximately 75% at an [EMIM]Ac/DMAc ratio of 3:7. However, these values were noticeably higher than those obtained at pretreatment temperatures of 80 and 100 °C.

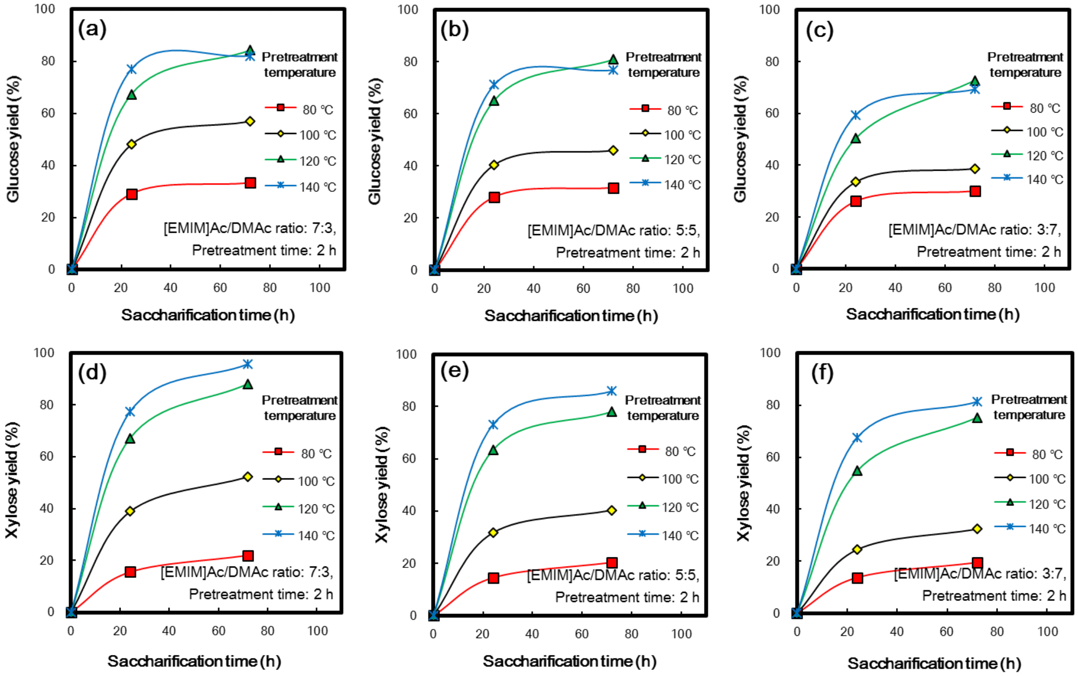

Fig. 6. Effect of the pretreatment temperature on enzymatic saccharification at [EMIM]Ac/DMAc ratio 7:3 (a, d), 5:5 (b, e), and 3:7 (c, f)

CONCLUSIONS

1. The addition of DMAc to [EMIM]Ac improved not only the mixing efficiency due to its lower viscosity, but its effect on the improvement of enzymatic saccharification was not significant, compared to the pretreatment using only [EMIM]Ac.
2. The yields of water soluble fraction by co-solvent were slightly lower than the that obtained using [EMIM]Ac alone, and there was no significant effect of the pretreatment time on the amount of water soluble fraction at 120 °C. The amount of water soluble fraction obtained at 140 °C was the greatest in all [EMIM]Ac/DMAc ratios.
3. The cellulose crystallinity decreased with increasing amount of [EMIM]Ac in the co-solvent and increasing pretreatment temperature, exhibiting a nearly amorphous pattern under [EMIM]Ac amounts greater than 50% and temperatures higher than 120 °C.

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