

# The combined effect of inorganic salt and ionic liquid in pretreatment on enzymatic saccharification of rice straw

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**Abstract.** The pretreatment method is one of the challenging steps in the production of biofuel through the biorefinery process that unlocks the recalcitrant nature of lignocellulosic biomass. Ionic liquid pretreatment gained attention for being highly effective to improve the enzymatic saccharification of the biomass, however its high cost hinders its industrial application. In this study, the combined effect of ionic liquid 1-ethyl-3-methylimidazolium acetate (EMIM-Ac) with inorganic salts (NaCl and KCl) was used for the pretreatment of rice straw. Optimization of pretreatment was conducted based on Response Surface Methodology and sugar yields obtained by EMIM-Ac+NaCl (160 °C, 88.7 min, 7.6%wt) and EMIM-Ac+KCl (160 °C, 68.2 min, 12.5%wt) were 670.7 and 392.9 mg/g-biomass, respectively. The effect of combined pretreatment on ethanol production was analyzed after 48h fermentation. The results showed that the ethanol yield from pretreated samples with EMIM-Ac+NaCl (0.72%) and EMIM+KCl (0.76%) was increased by 2.18 and 2.25 fold times, respectively, compared to untreated sample (0.33%). This combined effect of inorganic salts and ionic liquid significantly removed the lignin during pretreatment, while maintaining efficient enzymatic saccharification of rice straw. Thus, this cost-effective combined chemical method may be an alternative strategy for increasing cellulosic ethanol production.

**Keyword.** Biorefinery, Enzymatic saccharification, Inorganic salt, Ionic liquid, Lignocellulose, Pretreatment

## 1 Introduction

Due to intensive awareness of global society on the global warming effects, the concept of Bioeconomy, Circular economy and Green economy (BCG economy) has been implemented for manufacturing and industrialization. The use of fossil fuels as raw materials for the production of fuels and chemicals can be substituted by using various types of biomass. Lignocellulose biomass is acknowledged as the most abundant biomass on earth. After the harvesting season, lignocellulose biomass in agricultural waste is burned on fields to clear the land for the next season and air pollution was uncontrolled released into the environment, especially PM<sub>2.5</sub> and PM<sub>10</sub> [1]. Therefore, it is important to utilize lignocellulose biomass in the controllable system to minimize the negative impact to the environment and it has the potential to produce value-added products (e.g. biofuels, biochemicals, platform chemicals) to gain more benefit to the process [2-5].

To efficiently convert the lignocellulose biomass to biofuels, a multi-step process, including pretreatment, hydrolysis, fermentation and product recovery/separation, has to be conducted [6]. Pretreatment has been proved in many studies to be critical to determine the success of the process, in terms

of efficiency and economy by improvement of enzymatic hydrolysis. The functions of pretreatment include i) modifying cellulose fibril to be more vulnerable to enzymatic saccharification, ii) increasing surface area to allow enzyme accessibility, iii) removing hemicellulose and lignin content [7]. Pretreatment methods are mainly classified to be chemical, physical and biological methods. The selection of the appropriate pretreatment method for each type of biomass requires research and preliminary investigation to maximize the process efficiency with less complication and investment.

Among various types of chemical pretreatments, ionic liquids (ILs) have been demonstrated to be green solvents with high efficiency for the improvement of enzymatic saccharifications of lignocellulose biomass [8]. IL is a salt composed of cation and anion, and it mostly has a liquid state at room temperature or below 100 °C. ILs have various properties that are suitable for the biorefining process, such as high polarity, low volatility, low vapor pressure, heat stability, low melting point, and recyclability. The most common ILs derivatives used in lignocellulose pretreatment are imidazolium, choline, and ammonium, pyridinium, etc. [9]. ILs function in fractionation and solubilization of cellulose and lignin, as well as modification of cellulose arrangement to promote the enzyme accessibility to

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biomass during hydrolysis reaction [10-12]. Although IL is an efficient chemical for pretreatment, it has a high cost, which makes it infeasible for industrial applications.

This study, the inorganic salts were formulated with an IL, 1-ethyl-3-methylimidazolium acetate (EMIM-Ac), to reduce the fraction of IL for pretreatment and the cost. Previously, inorganic salts, NaCl, KCl, MgCl<sub>2</sub>, NaNO<sub>3</sub>, KNO<sub>3</sub>, Mg(NO<sub>3</sub>)<sub>2</sub>, were used in the pretreatment of rice straw biomass, and the yields of reducing sugars were improved for more than 4.7 times when using NaCl and KCl [13]. The optimization of pretreatment with mixed EMIM-Ac and NaCl and KCl was conducted based on Response Surface Methodology (RSM) to maximize the sugar yields obtained from cellulase saccharification. The hydrolysates were then converted to ethanol to assess the potential of biomass for biofuel production.

## 2 Materials and methods

### 2.1. Material preparation and chemicals

Rice straw samples were harvested after the harvesting season from a local rice field located in the central part of Thailand. The size of the sample was reduced by hand cutting to about 1-cm long pieces and dried in a hot air oven at 60 °C until the dried weight was obtained. To obtain a consistent sample size, dried rice straw was ground by blender and sieved through a 20-mesh aluminium sieve. The prepared biomass was kept in a sealed plastic bag until used. EMIM-Ac and CelluClast 1.5L were purchased from Sigma-Aldrich. Other chemicals, including inorganic salts, DNS, and glucose were purchased Ajax Finechem.

### 2.2. Optimization of pretreatment

The EMIM-Ac and inorganic salts (NaCl and KCl) were pre-mixed with the ratio of 1:10 by weight. The pretreatment experiment was designed based on Box-Behnken by using Design Expert Software (Version 7.0.0, Stat-Ease) [14-15] to target to vary the three pretreatment factors, including pretreatment temperature (X<sub>1</sub>: 100-160 °C), pretreatment time (X<sub>2</sub>: 60-180 min) and biomass loading ratio (X<sub>3</sub>: 5-15%wt). A total of 17 experimental runs (Table 1) were designed and each pretreatment run was conducted. Then, each pretreated sample was enzymatic hydrolyzed and the reducing sugar yield was analyzed. The influences of pretreatment factors and interacting factors were evaluated based on ANOVA. The mathematical model to represent the correlation of pretreatment factors and sugar yields was constructed and the reliability of the model was assessed based on the R<sup>2</sup> value and p-value.

To pretreat the rice straw sample, the pre-mixed solution (EMIM-Ac and inorganic salts) was added to rice straw in different ratios of biomass loading (5-15%wt). Then, the mixture was placed in a hot air oven at the targeted pretreatment temperature and time. After pretreatment, the sample was cooled down to room temperature. Then, the solid fraction of the sample was collected by centrifugation at 8,000 xg for 10 min. The pretreated sample was washed with distilled water three

times to remove IL and salt residues. The washed solid sample was subjected to enzymatic saccharification.

### 2.3. Enzymatic saccharification

The pretreated solid sample was loaded at the loading ratio of 2.5%wt in 4 ml of hydrolysis buffer (containing 20 FPU/g-biomass, 40 ul of 2% sodium azide in 50 mM sodium citrate buffer, pH 4.8). The mixture was placed in a shaking incubator at 45 °C, 200 rpm, for 72 h. After 72 h, the progress of the reaction was stopped by heating in the waterbath at 100 °C for 5 min. The supernatant fraction was collected from rice straw hydrolysate by centrifugation at 8,000 xg for 10 min [16-17]. The reducing sugar concentration in the hydrolysate was measured by using a DNS assay. All measurements were repeated three times.

### 2.4. Ethanol fermentation

The liquid fraction of hydrolysate (without the addition of sodium azide) was subjected to fermentation for ethanol production using yeast, *Saccharomyces cerevisiae* TISTR 5606 [18-19]. For each experiment, 5% of yeast inoculum (at the concentration of about 10<sup>8</sup> cells/ml) was added to 19 ml of rice straw hydrolysate supplemented with 1%w/v glucose and 1% w/v yeast extract [18, 19]. The sample was incubated in a shaking incubator at 30 °C, 150 rpm for 72 h. Then, the supernatant fraction of yeast culture was separated by centrifugation at 8,000 xg for 10 min. The ethanol concentration in the supernatant fraction was measured by using our published protocol based on spectrophotometry [20]. All measurements were conducted in triplicates to confirm the reliability of the analysis.

### 2.5. FT-IR analysis

The chemical modification of rice straw by IL-inorganic salt pretreatment was assessed by using Fourier transform infrared (FT-IR) spectrometry. The pretreated and untreated samples were finely ground before analysis. The absorption spectra were scanned with a resolution of 4 cm<sup>-1</sup> in the range of 400–1800 cm<sup>-1</sup>. The spectral data were developed by Spectrum 2.00 software (Perkin Elmer, USA).

## 3 Results and discussion

### 3.1. Optimization of pretreatment by EMIM-Ac and inorganic salts

The pretreatment experiments were designed based on RSM by varying 3 pretreatment factors, including pretreatment temperature, pretreatment time and biomass loading ratio. After pretreatment, biomass was enzymatically hydrolyzed and reducing sugars were quantitated. In this study, the ranges of reducing sugar yields were between 86.7 (run 10) and 526.2 (run 7) mg/g-biomass, which was about 6 fold time difference (Table 1). This range suggested the importance of optimization to improve the pretreatment efficiency.

The effects of each pretreatment factor and interacting factor on reducing sugar yields were analyzed

**Table 1.** Experimental design of pretreatment by EMIM-Ac and inorganic salts based on RSM.

Run	Pretreatment condition			Reducing sugar yield (mg/g-biomass)	
	Temperature (°C)	Time (min)	Biomass loading (%wt)	EMIM-Ac+NaCl	EMIM-Ac+KCl
1	130	120	10	407.1	283.3
2	160	60	10	758.2	451.0
3	160	120	15	761.9	421.8
4	130	180	15	420.8	214.3
5	130	120	10	378.2	203.0
6	160	120	5	618.3	237.4
7	160	180	10	663.7	526.2
8	130	60	15	282.3	142.4
9	130	120	10	490.7	212.4
10	100	120	15	277.1	84.8
11	130	120	10	387.7	151.4
12	130	180	5	461.0	265.8
13	100	60	10	171.4	86.7
14	100	180	10	191.6	117.4
15	130	60	5	300.8	222.3
16	100	120	5	183.1	128.8
17	130	120	10	448.6	227.0

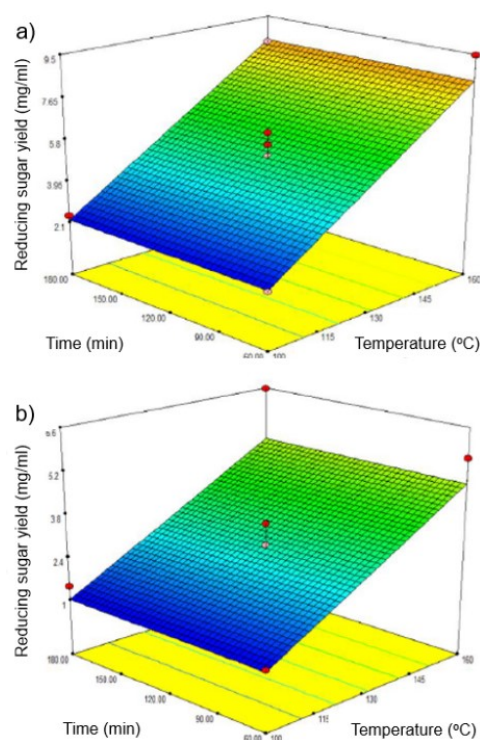
based on ANOVA analysis and mathematical models were generated. The model significance levels of both EMIM-Ac+NaCl and EMIM-Ac+KCl pretreatments were calculated to be P-value <0.0001. The correlation efficiency ( $R^2$ ) values are 0.8864 and 0.8811, respectively, advocating the reliability of the generated models [21]. The mathematic models obtained from both EMIM-Ac+NaCl and EMIM-Ac+KCl were presented as shown in eq 1 and eq 2, respectively.

$$\text{Reducing sugar yield (mg/0.1g-biomass)} = -8.10267 + 0.10307 \times \text{temperature} \quad (\text{eq 1})$$

$$\text{Reducing sugar yield (mg/0.1g-biomass)} = -5.32690 + 0.063467 \times \text{temperature} \quad (\text{eq 2})$$

The impacts of pretreatment factors on reducing sugar yields were visualized in 3D models to observe the impacts of two pretreatment factors at a time (Figure 1). It could be seen that the pretreatment factor was the significant parameter for reducing sugars. The increasing of pretreatment temperature improves the reducing sugar yields. However, in this study, the pretreatment time and biomass loading ratio were not significant factors influencing sugar yields, which may be the result of an inappropriate range of pretreatment factors.

Based on the RSM model, the optimal pretreatment conditions to maximize the sugar yields were predicted for both EMIM-Ac+NaCl (160 °C, 88.7 min, 7.6%wt) and EMIM-Ac+KCl (160 °C, 68.2 min, 12.5%wt) and the optimal sugar yields were predicted to be 670.7, and 392.9 mg/g-biomass, respectively (Table 2). To verify the reliability of the predicted mathematic models obtained from RSM, the pretreatments at optimal conditions were conducted and the reducing sugar yields were measured and compared with the yields obtained from the predicted model. The sugar yields obtained from the pretreatment by EMIM-Ac+NaCl and EMIM-Ac+KCl at the optimal condition were 670.7 and 392.9 mg/g-biomass, respectively (Table 2), which were only 0.04% and 1.73% difference compared to the predicted



**Figure 1.** 3D models representing the correlation of pretreatment factors on reducing sugar obtained from a) EMIM-Ac+NaCl pretreated sample and b) EMIM-Ac+KCl pretreated sample.

yields. This result proves the validity of the RSM model of pretreatment. It should be noted that both pretreatments improved the reducing sugar yields by 2.24 and 1.32 fold times, compared to the untreated sample (298.1 mg/g-biomass).

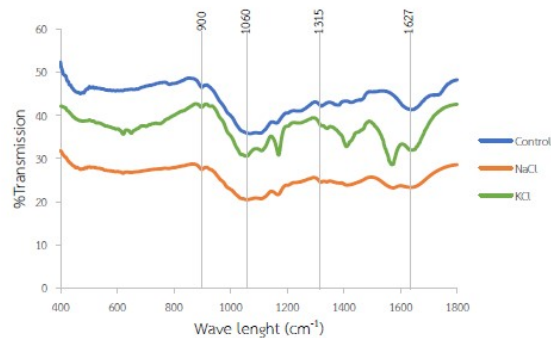
**Table 2.** Predicted pretreatment conditions and reducing sugar yields obtained from the RSM model.

Chemical	Pretreatment condition			Reducing sugar yield (mg/g-biomass)	
	Temperature (°C)	Time (min)	Biomass loading (%wt)	Predicted value	Experimental value
EMIM-Ac+NaCl	160	88.7	7.6	671.0	670.7
EMIM-Ac+KCl	160	68.2	12.5	386.2	392.9

### 3.2. Microscopic chemical analysis of pretreated rice straw

To evaluate the effect of pretreatment on lignocellulose biomass, the arrangements of chemical bonds in biomass were analyzed by using FT-IR spectrometry. The spectra peaks that are corresponding to the chemical bonds in cellulose, hemicellulose and lignin and the linkages between these compounds were selectively analyzed (Figure 2). The spectral peaks at 900  $\text{cm}^{-1}$  and 1060  $\text{cm}^{-1}$  representing  $\beta$ -glucosidic bond and C=O stretching bond in cellulose fibrils were increased in pretreated samples, suggesting enriched cellulose contents [22]. The height of the peak at 1315  $\text{cm}^{-1}$ , assigned intermolecular hydrogen bond in amorphous cellulose, was also increased in pretreated samples, suggesting the

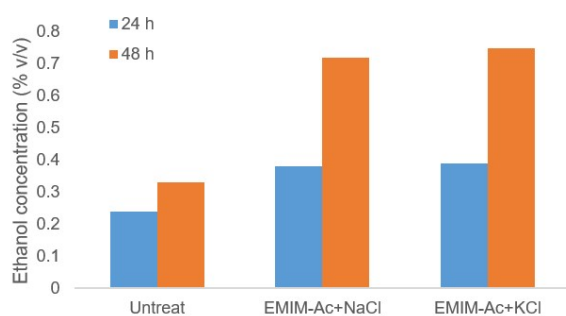
enhancement of enzymatic saccharification. On the other hand, the peak height at 1627  $\text{cm}^{-1}$ , representing C=C bond in an aromatic skeleton of lignin [23-24], was reduced in pretreated samples, suggesting the removal of lignin from biomass during pretreatment.



**Figure 2.** FT-IR spectra observed from untreated and pretreated rice straw

### 3.3. Effect of pretreatment by combined EMIM-Ac and inorganic salts on ethanol production

To evaluate the efficiency of pretreatment of lignocellulose for application in the biorefining process, the hydrolysates obtained from untreated and pretreated biomass were converted to ethanol by fermentation with *S. cerevisiae* TISTR 5606. Within 48 h fermentation time, the ethanol concentrations in fermented hydrolysates were analyzed. The results showed that the ethanol yields at 48 h obtained from pretreated samples by EMIM-Ac+NaCl (0.72%) and EMIM+KCl (0.76%) were increased by 2.18 and 2.25 fold times, respectively, compared to untreated sample (0.33%) (Figure 3).



**Figure 3.** Ethanol production from untreated and pretreated rice straw by *S. cerevisiae* TISTR5606

It should be noted that the increases in ethanol yields by EMIM-Ac+NaCl and EMIM-Ac+KCl were similar, while the increase in sugar yield caused by EMIM-Ac+NaCl pretreatment was higher compared to EMIM-Ac+KCl (Table 2). This trend for the improvement of sugar yield production by NaCl and KCl was also demonstrated in the previous study that NaCl was the better chemical for rice straw pretreatment compared to KCl [13]. Here, when these two inorganic salts were formulated with EMIM-Ac, a similar effect on sugar yield was observed. It could be hypothesized that Na<sup>+</sup> has high ionic strength compared to K<sup>+</sup>, which could

interfere with the arrangements of intermolecular bonding of cellulose fibrils and improve the disintegration of cellulose during saccharification. However, although the sugar yield obtained from EMIM-Ac+NaCl pretreated sample was higher than EMIM-Ac+KCl pretreated sample, the ethanol yields of both samples were similar. In previous studies, the inhibitory effects of NaCl and KCl on cellulase enzyme were observed during enzymatic saccharification [25]. Testing with the same concentration, the enzyme activities were reduced with a higher degree by KCl compared to NaCl. So, it is a plausible cause for ethanol production trend observed in this study.

## 4 Conclusion

The synergistic effect of inorganic salt and IL on the enzymatic saccharification of rice straw was studied. Optimization study using RSM generated higher yields of reducing sugars using rice straw biomass. Increased intermolecular H-bonding and reduction in the peak positions (C=C) in regions representing aromatic lignin structures suggest decreased recalcitrance by lignocellulosic biomass structure. Na<sup>+</sup> ions having higher ionic strength than K<sup>+</sup> gave higher reducing sugar yields using EMIM-Ac+NaCl in comparison to that obtained by pretreatment using EMIM-Ac+KCl. Ethanol yield of pretreated samples increased by using EMIM-Ac+NaCl (0.72%) and EMIM+KCl (0.76%) by 2.18 and 2.25 fold, respectively. The present work thus suggests a combined pretreatment study targeted at an enhanced yield of reducing sugars and ethanol using rice straw.

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