Improvement of potassium permanganate pretreatment by enzymatic saccharification of rice straw for production of biofuels

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Abstract. Commonly, the agricultural waste, i.e. lignocellulosic biomass is disposed through combustion causing air pollution with production of PM2.5 and PM10 particles. However, it has been found that these biomasses can be used as source for the production of biofuels and other valuable biochemicals. Though deconstruction of lignocellulosic biomass is challenging due to its complex structure. In this study, rice straw (RS) was pretreated using potassium permanganate ($KMnO_4$) to enhance the enzymatic saccharification efficiency. The study was carried out by varying the operational factors in pretreatment, including temperature (30-90°C), time (30-360 min) and concentration of $KMnO_4$ (0.5-3.0, % w/v), respectively, based on Box-Behnken design (BBD). Through multi-regression analysis of the experimental data obtained after pretreatment, the optimum conditions were determined. The optimum conditions for temperature, time and potassium permanganate concentration were 48.09°C, 360 min, and 1.36% w/v, respectively. The saccharifications of pretreatment and untreated rice straw were carried out using Cellic Ctec2. The reducing sugar was determined by using DNS method and the yields of the untreated and pretreated RS were 32.38 and 49.011 mg/mL, respectively. The results showed that the sugar for pretreated RS were 1.51 fold times higher compared to untreated RS. Therefore, this work illustrates the pretreatment efficiency for KMnO4 to enhance the reducing sugar yield during saccharification, which can be used for biofuel and value-added product productions.

Keywords. Potassium permanganate, Pretreatment, Enzymatic saccharification, Biorefinery, Biofuels

1 Introduction

Lignocellulosic biomass is an inedible organic material source having the potential to be utilized as an alternative source for fossil fuels. Common lignocellulosic biomass includes forest residues, agricultural residues and dedicated crops. It has been reported in 2013 that 62 million tons of waste biomass are generated from the agricultural activities of Thailand. The biomass waste accounts for 13.3 million tons and is likely to increase further in coming years. Increase in the waste generation is related to the continuous increase in the food demand due to the rapid growth of population. The industrial growth is also proportional to the population growth but it greatly affects the environments [1]. It was reported that the air was highly polluted in Thailand (2007-2016) due to the presence of particulate matters (PM10 an PM2.5) [2]. One of the causal agents of these polluted particles was due to the incineration of waste biomass generated after the harvesting seasons. This tradition is being followed worldwide to prepare the soil for next harvesting season. Incinerations of agricultural residues also increase in the emission of carbon dioxide (CO_2), an important of greenhouse gas.

To lessen combustion of agricultural waste, lignocellulose biomass has been proposed to utilized as raw materials for productions of biofuels and biochemical [3]. Lignocellulose is composed of cellulose, hemicellulose, and lignin. Each of this component varies in different ratio from biomass to biomass. In general lignocellulose composes of cellulose, hemicellulose and lignin in the range of 40-50%, 20-30%, and 10-25%, respectively [4-5]. Cellulose is a polysaccharide chain of glucose units linked via β-

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1,4 glycosidic bonds. On the other hand, hemicellulose is a polysaccharide with C_5 sugars (xylose and arabinose) and C_6 sugars (glucose, galactose, and mannose). Lignin is an organic polymer composed of phenylpropane units. [6-8]. However due to the recalcitrance nature of the lignocellulosic biomass, it has been a challenge for the researchers for converting biomass to biofuel and other by-products. Conversion of lignocellulose to the valuable products, therefore requires three major steps such as pretreatment, saccharification, and fermentation. [9-11]

In recent years, several pretreatment methods have been developed for the fractionation of lignocellulosic biomass. Pretreatment methods include physical pretreatment (mechanical size reduction, pyrolysis), chemical pretreatment (ozonolysis, acid pretreatment, alkali pretreatment, oxidative delignification), physicochemical pretreatment (stream explosion, ammonia fiber explosion, microwave pretreatment, carbon dioxide explosion, wet oxidation) and biological pretreatment (fungus and bacteria) [4], [12], [13-17]. Pretreatment is an essential step prior to saccharification process. Pretreatment leads to fractionation of biomass through solubilization and structure modification of lignin and hemicellulose, respectively. After pretreatment, cellulose is converted to glucose by the cellulase enzymes due the ease in accessibility. Though pretreatment methods have advantages in the fractionation of biomass, each pretreatment method has its own disadvantage, for instance, some pretreatment methods require high energy or use toxic substances or have high operational cost [4].

Among the different chemical pretreatment methods, acid and alkali pretreatments are studied worldwide. However strong acid and alkaline are considered to be harmful to both the operator and the environment. In addition, acid pretreatment degrades sugars to inhibitory compounds (furfural and 5-hydrozymethylfurfural [18]) to the functions of enzymes, and therefore decreases the conversion efficiency from cellulose to glucose monomers. On the other hand, lignin and hemicellulose is solubilized completely or partially during alkaline pretreatment, respectively [17-20]. Lee et. al., reported delignification and saccharification efficiency of 89.4% and 75.9% in corn stover after diluted alkaline pretreatment [21]. On the other hand, Ravindran et. al., reported delignification and cellulose recovery of 46% and 98%, from $KMnO_4$ pretreated spent coffee waste, respectively [22]. Ma et. al., reported 46.79%, and 81.47% removal of lignin and hemicellulose from pretreated corncob using $KMnO_4$ [23].

In this study, $KMnO_4$ pretreatment of the rice straw was carried out to improve saccharification efficiency. $KMnO_4$ is inexpensive, readily available, and less toxic compared to acids such as sulphuric acid and it is one of the common chemicals in kitchen application. $KMnO_4$ pretreatment of rice straw was carried out by varying the reaction time, temperature and concentration of $KMnO_4$. The $KMnO_4$ pretreatment conditions were optimized based on Response Surface Methodology (RSM) with Box Behnken Design (BBD). The operational parameters were optimized through multiple regression analysis that generated the mathematical model. The optimal condition of pretreatment obtained from the model was validated by experiments to confirm the reliability of the predicted pretreatment conditions.

2 Material and methods

2.1. Raw materials

Rice straw was obtained from Phra Nakhon Si Ayutthaya province, Thailand. The collected rice straw was subjected to particle reduction using food processor after drying in a hot air oven at 60°C to remove moisture. The dried sample was screened through 20 mesh sized aluminium sieve for the homogeneous nature of particle size.

Cellic Ctec2, a commercial cellulase enzyme, was obtained from Sigma-Aldrich (aqueous solution, density 1.15 g/mol). This enzyme is a cocktail mixture of cellulases, ß-glucosidases and hemicellulase. Potassium permanganate ($KMnO_4$), and other analytical reagents were obtained from Alex Finechem (Univar).

2.2 Pretreatment of rice straw

Pretreatment of rice straw using $KMnO_4$ was carried out under different reaction time, temperature and concentration of $KMnO_4$. A solid loading of 10% (1:10 w/w) was maintained throughout the study. Firstly, the testing ranges of pretreatment factors, such as reaction time, temperature, and concentration of $KMnO_4$ were varied to 2-8 h, 30 – 75°C, and 1 – 4% w/v, respectively to preliminarily evaluate the boundary of testing ranges for RSM experiments. Secondly, after the determination of the suitable testing ranges for RSM, the experimental designed for optimization was carried out based on BBD. Three independent variables, composing of time (30-360 min), temperature (30 - 90°C), and $KMnO_4$ concentration (0.5 - 3% w/v), were set with three testing levels (-1, 0, and +1) as summarized in Table 1. The pretreatments of rice straw were carried out by following the individual runs as summarized in Table 2. The pretreated solid biomass was harvested by filtration with No.1. Whatman filter paper to remove the liquid hydrolysate. After collection of the hydrolysate, the solids were washed using deionized water until pH was neutral. The pretreated solids were dried in a hot air oven at 60°C until the weight was constant. The dried pretreated and untreated samples were subjected to enzymatic saccharification, and reducing sugars released from hydrolysis reactions were analysed by DNS assay to determine the pretreatment efficiency.

The amounts of reducing sugars obtained from each experimental runs were analyzed by ANOVA analysis to observe the effect of pretreatment parameters on yields of reducing sugars with the significant level of P-value <0.05 for the model and term model. The mathematical model derived from ANOVA analysis was then generated to predict the optimal pretreament condition. The correlation coefficiency (R^2) was calculated to confirm the reliability of the predicted model. All statistical analysis of the experimental results obtained for the BBD design were analyzed using Design-Expert software (version 7.0)

Table 1. Independent variables in pretreatment condition of rice straw based on RSM.

Independent variable	**Coded symbols**	**Levels**		
		-1	**0**	**+1**
Time (min)	X_1	30	195	360
Temperature (°C)	X_2	30	60	90
Concentration (%)	X_3	0.50	1.75	3.00

Table 2. RSM experiment design for $KMnO_4$ pretreatment of rice straw

Run	**Pretreatment condition**			**Reducing sugar (mg/mL)**
	X_1: Time (min)	**X_2: Temperature (°C)**	**X_3: Concentration (%)**	
1	195	60	1.75	47.86
2	195	30	0.50	43.36
3	360	60	3.00	30.53
4	30	60	0.50	42.60
5	360	90	1.75	52.70
6	360	60	0.50	45.25
7	30	30	1.75	43.99
8	195	60	1.75	47.86
9	195	30	3.00	31.25
10	195	60	1.75	45.59
11	30	60	3.00	31.61
12	195	60	1.75	51.02
13	195	90	0.50	45.72
14	30	90	1.75	46.68
15	360	30	1.75	49/04
16	195	60	1.75	49.75
17	195	90	3.00	30.36

2.3 Enzymatic hydrolysis

Enzymatic saccharification process was carried out for untreated and pretreated rice straw to determine the efficiency of $KMnO_4$ pretreatment. For each reaction, 0.2 g of the biomass (untreated and pretreated) was added to 8 mL of citrate buffer (50 mM, pH 4.8) in a 50 mL centrifuge tube. In addition, 80 µL of 2 M sodium azide were added to avoid the contamination of the samples due to growth of microbes during saccharification process. To the mixture, 1.4 µL Cellic Ctec2 enzyme mix was added. The mixed samples were placed in a shaking incubator with the temperature and mixing speed at 50°C and 150 rpm, respectively. Enzymatic saccharifications of the untreated and treated rice straw were carried out for 72 h. After 72 h, the liquid hydrolysate was taken from the mixture by centrifugation at 10,000×g for 5 min. The supernatant was collected for the determination of the reducing sugar by following the modified dinitrosalicylic acid (DNS) method.

2.4 Analysis of reducing sugar

The reducing sugar concentration in the hydrolysate was measured by following the dinitrosalicylic acid (DNS) method. In a 1.5 mL centrifuge tube, 50 µL of hydrolysate and 3,5-dinitrosalicylic acid (DNS) reagent was added. The tubes were incubated at 95°C using a water bath for 5 min. The tubes were allowed to cooled down in an ice bath for 5 min. After cooling of the sample, 1 mL of distilled water was added and mixed with a vortex shaker. After the samples were prepared, the reducing sugar was analyzed using UV/Vis spectrophotometer at 540 nm. The concentration of the reducing sugar (mg/mL) was calculated by using a standard glucose curve.

3 Result and discussion

3.1 Determine the testing levels of pretreatment parameters

The effects of reaction, temperature and $KMnO_4$ concentration on pretreatment of rice straw were preliminary evaluated to set the boundary of testing level for RSM. Three sets of experiments to pretreat rice straw were conducted by varying each pretreatment factor at a time (Figure 1). Firstly, the pretreatment time was varied between 2 to 8 hr, and the pretreatment temperature was fixed at 40°C using 2% (w/v) $KMnO_4$ (Figure 1a). Secondly, the pretreatment temperature was varied between 30 to 75°C, and the pretreatment time was fixed at 4 h, using 2% (w/v) $KMnO_4$ (Figure 1b). Thirdly, the $KMnO_4$ concentration was varied between 1-4%, and the pretreatment time and temperature were set at 40°C for 4 h (Figure 1c). It was observed from Fig. 1a that the reducing sugar yield varied for pretreated samples at different reaction time (2 – 8h). The reducing sugar yields increased 1.15 fold times (18.36 to 21.13 mg/mL), when the reaction time was varied between (2-6 h) and this increasing yield was saturated at between 6-8 h. Therefore, the upper boundary level of pretreatment time of 6 h was selected for RSM experiments. Next, the testing boundary level of pretreatment temperature (30–75°C) on saccharification efficiency was evaluated (Fig. 1b). Maximum reducing sugar yield of 23.68 mg/mL was obtained when rice straw was pretreated at 60°C. As the pretreatment temperature was increased to 75°C, it was found that the reducing sugar yield decreased 1.01 fold times. Therefore, it was implied as similar to the effect of reaction time that, sugars were degraded at higher temperature. Due to the lower effect of temperature on reducing sugar from rice straw pretreated at 75°C. The temperature for BBD design was considered to be 30 – 90°C. Meanwhile, the independent effect of KMNO4 concentration on reducing sugar yield during saccharification of pretreated rice straw was also investigated (Fig.1c). The concentration of $KMNO_4$ was varied from 1-4% w/v. It was noted that the reducing sugar yield was higher initially (27.04 mg/mL), when the $KMnO_4$ concentration was maintained at 1% w/v

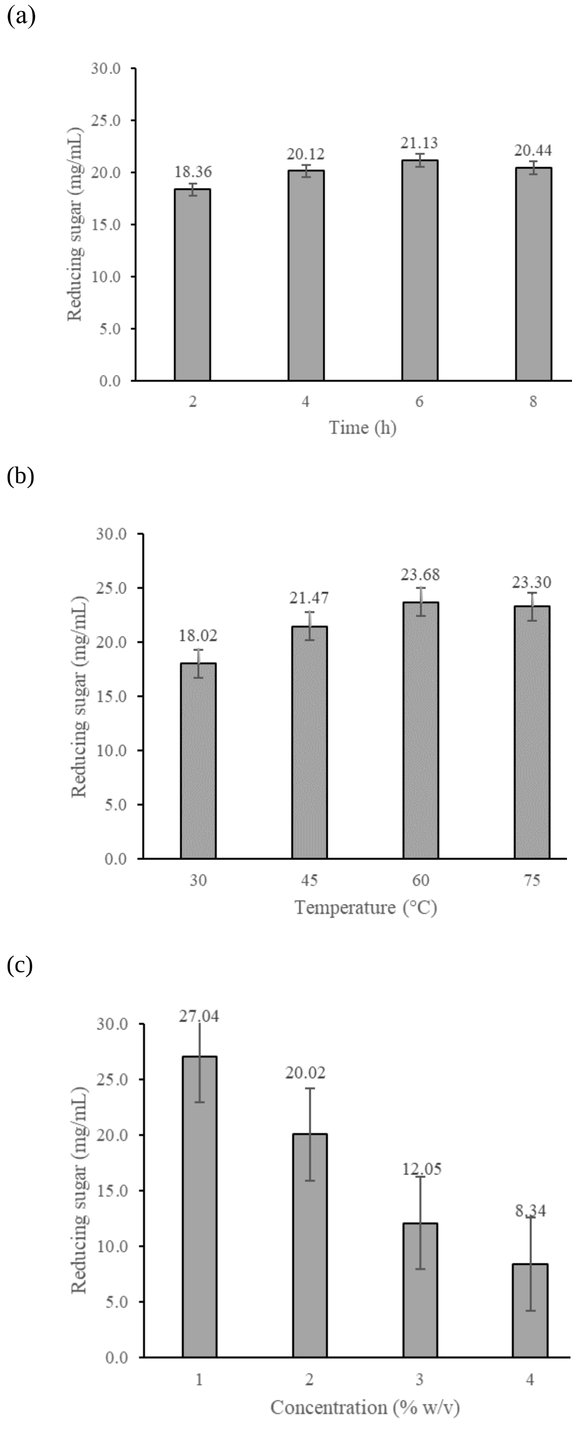

Fig. 1. Effect of pretreatment time, temperature and $KMnO_4$ concentration on the enzymatic hydrolysis of rice straw. (A) The pretreatment was conducted using 2% (w/v) $KMnO_4$ at 40°C, (B) The pretreatment was conducted using 2% (w/v) for 4 hours and (C) The pretreatment were conducted at 40°C for 4 hours.

However, as the $KMNO_4$ concentration increased from 1 – 4% w/v, the reducing sugar yield decreased 3.24 fold times (27.04-8.34 mg/mL). Thus, the saccharification efficiency was negatively impacted by the increase in concentration of $KMnO_4$ during pretreatment. Therefore, the $KMnO_4$ concentration in the BBD design was varied from 0.5-3.0% w/v. The results or released reducing sugars showed that the parameters with the preliminary tested ranges were not statistically significant. Therefore, the independent variables levels were modified and used in the BBD design of RSM study (Table 1).

Overall, it was observed that the testing levels of independent variables (pretreatment time, pretreatment temperature and $KMnO_4$ concentration) had effects on enzymatic saccharifications of rice straw. This finding was in agreement with different studies carried out by several published works. Romero et al performed pretreatment of rapeseed straw using $FeCl_3$ under various conditions of time, temperature and $FeCl_3$ concentration. A direct correlation between reducing sugar content and operational parameters were reported [24]. In addition, Dahunsi et al performed alkali pretreatment of pineapple peel and reported similar correlation between reducing sugar contents and operational parameters [25].

3.2 RSM design and experimental testing

The RSM experiment has been proposed as an effective method of designing and conducting experiments to determine the optimal condition for production process. The RSM study assists evaluation of effects of individual independent variables and their interactions on the dependent variables. RSM reduces the numbers of experimental runs, in turn, reducing the experimental cost. Furthermore, RSM is often used for generation of the empirical model and prediction of the desired response under different conditions of independent variables [15]. In recent years, several studies have illustrated the significance of using RSM in reducing the experimental runs and improving the process efficiency by varying numbers of parameters conditions simultaneously [26-27].

In the present study, the RSM study was carried out by generating a BBD matrix (Table 3) with three testing levels (-1, 0, +1) for three pretreatment parameters (time, temperature and $KMnO_4$ concentration) (Table 1) with a total 17 experimental runs (Table 2). The statistical analysis was carried out using Design-Expert software (ver 7.0.0) throughout the whole study. The experimental data were used in determining the regression coefficient (β) using least square method. In addition, the second order model was generated from the experimental data. The optimum pretreatment conditions were determined for achieving maximum reducing sugar yield from the multi-regression analysis of the second-order model. The second order model significance were evaluated by (a) determining the coefficient of determination (R^2), (b) significance of model terms in ANOVA, and (c) non-significance of the lack-of-fit. The second-order model is generally considered to be significant when the R^2 value is greater than 0.9. In this study, the R^2 value of the second-order model was 0.9646 implying lower variance between the experimental data and the representative model. In fact, the R^2 value of higher than 0.9 does not always indicate the significance of the model. Therefore, ANOVA was carried out to determine the significance of the second-order model (Table 3).

Table 3. ANOVA analysis of the second-order model obtained from RSM experiments.

Source	Sum of Squares	df	Mean Squares	F Value	P-value Prob > F
Model	855.98	3	285.33	80.13	< 0.0001
A-Time	19.77	1	19.77	5.55	0.0348
C-Conc	352.76	1	352.76	99.07	< 0.0001
C^2	483.46	1	483.46	135.76	< 0.0001
Residual	46.29	13	3.56		
Lack of Fit	29.14	9	3.24	0.76	0.6677
Pure Error	17.15	4	4.29		
Cor Total	902.27	16			

The ANOVA results indicated that the model with F-value of 80.13 was statistically significant ($P<0.05$). There was only a 0.01% chance that a "Model F-value" could occur due to noise (Table 3). Furthermore, the model terms of time, and $KMnO_4$ concentration were also significant (since $P<0.05$). Based on the same criteria, it was noted that neither the interaction terms for time, temperature and $KMnO_4$ concentration was significant. However, significance for the quadratic term of $KMnO_4$ concentration was seen. Therefore, the accuracy of the second-order model was improved by including the model terms that were significant. Additionally, the model significance was also evaluated through determination of the lack-of-fit test. The F-value of 0.76 implied that the lack of fit is not significant relative to the pure error. There is a 66.77% chance that a "Lack of Fit F-value" this large could occur due to noise. Therefore, the second order model was considered significant since the lack of fit was not significant and the errors were normally distributed. Therefore, through evaluation of the second-order model, time and $KMnO_4$ concentration were considered to have more effect on the reducing sugar yield released during saccharification after pretreatment of rice straw.

3.3 Effects of pretreatment parameters on enzymatic saccahrification and validation of mathematical models for pretreatment

The effect of each significant model terms (time, and $KMnO_4$ concentration) on response (reducing sugar concentration) was monitored (Fig. 2). In addition, the interaction effects of the independent variables on response were visualized through three-dimensional contour plots (Fig. 3). It can be seen that the reducing sugar yield obtained from saccharification was increased when rice straw was pretreated with longer pretreatment time (Fig 2a). Under variations of $KMnO_4$ concentration, the reducing sugar yield was increased when the $KMnO_4$ concentration increased. However, when $KMnO_4$ concentration was higher than 1.36%, the more $KMnO_4$ concentration, the less reducing sugar yields (Fig 2b). Meanwhile, interaction effects of time, temperature, and $KMnO_4$ resulted in similar observation (Fig 3).

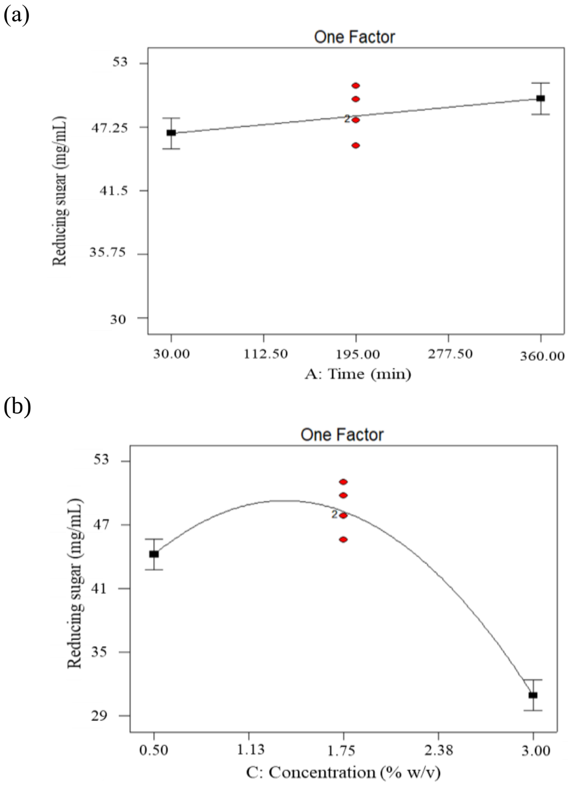

Fig. 2. The relationship between each pretreatment factor, including (A) pretreatment time (min) and (B) $KMnO_4$ concentration (%) and reducing sugar concentration (mg/mL) obtained from pretreated rice straw.

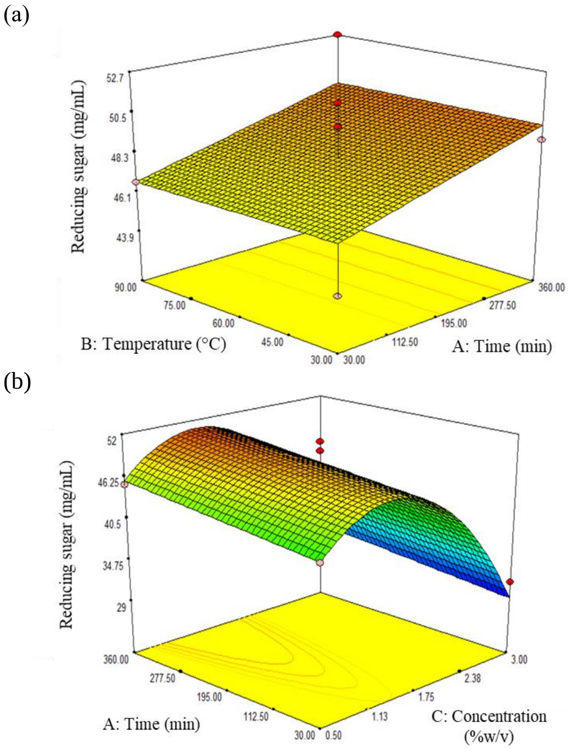

Fig. 3. Response surface plot represents the effects of pretreatment factors on reducing sugar yield (mg/mL). (A) pretreatment time vs. pretreatment temperature and (B) $KMnO_4$ concentration vs pretreatment time.

Through ANOVA analysis of the second order model, the relationships of pretreatment conditions on reducing sugar concentrations were represented in the form of mathematical model (Table 4). The optimum pretreatment conditions for obtaining maximum reducing sugar yield at 50.88 mg/mL were predicted and summarized in Table 4. To confirm the reliability of this model, the rice straw was pretreated again using this predicted optimum pretreatment conditions. A reducing sugar yield of 49.01 mg/mL was obtained from the enzymatic saccharification of the pretreated rice straw under optimum pretreatment conditions. Thus, there was only 3.49% error obtained from experimental result compared to predicted yield from the model, indicating high validity of RSM model. The influence of time and $KMnO_4$ concentration on reducing sugar yield were in agreement with studies carried out by Romero at al. [24] and Zhao et al. [28]. Therefore, it was found that increase in reaction time with optimum $KMnO_4$ concentration can improve enzymatic saccharification efficiency.

Table 4. Mathematical models and optimal pretreatment condition obtained from RSM experiment.

Optimal pretreatment parameter			**Reducing sugar yield (mg/mL)**	
Time (min)	**Temperature (°C)**	**$KMnO_4$ Concentration (% w/v)**	**Predicted**	**Experimental**
360	84.09	1.36	50.88	49.01
Mathematical Model Reducing Sugar = +34.77502 + 9.52745 × 10-3 × Time + 18.62003 × Conc – 6.83781 × Conc^2				

4 Conclusion

The present study was focused on the objective to obtain maximum reducing sugar from rice straw during saccharification after pretreatment using $KMnO_4$. These reducing sugars have the potential to be converted to biofuels and other valuable products. The individual effect of the operational parameters (time, temperature and $KMnO_4$ concentration) was studied based on RSM with BBD. The optimum pretreatment conditions for time, temperature, and $KMnO_4$ concentration were 84.09ºC, 360 min, and 1.36% (concentration of $KMnO_4$). The maximum reducing sugar yield from pretreated rice straw under optimum conditions were 49.01 mg/mL. In conclusion, $KMnO_4$ pretreatment had significant effect on rice straw to improve the saccharification efficiency and had potential to be applied in further uses due to its less toxicity and cost.

Acknowledgement

The authors would like to thank the King Mongkut's University of Technology North Bangkok (Grant Contract No. KMUTNB-BasicR-64-37) for financial support of this work.

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