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Optimization of Diluted Organic Acid Pretreatment on Rice Straw Using Response Surface Methodology

Rotchanaphan Hengaroonprasan, Malinee Sriariyanun, Prapakorn Tantayotai, Supacharee Roddecha, Kraipat Cheenkachorn

***Abstract*—**Lignocellolusic material is a substance that is resistant to be degraded by microorganisms or hydrolysis enzymes. To be used as materials for biofuel production, it needs pretreatment process to improve efficiency of hydrolysis. In this work, chemical pretreatments on rice straw using three diluted organic acids, including acetic acid, citric acid, oxalic acid, were optimized. Using Response Surface Methodology (RSM), the effect of three pretreatment parameters, acid concentration, treatment time, and reaction temperature, on pretreatment efficiency were statistically evaluated. The results indicated that dilute oxalic acid pretreatment led to the highest enhancement of enzymatic saccharification by commercial cellulase and yielded sugar up to 10.67 mg/ml when using 5.04% oxalic acid at 137.11^{o}C for 30.01 min. Compared to other acid pretreatment by acetic acid, citric acid, and hydrochloric acid, the maximum sugar yields are 7.07, 6.30, and 8.53 mg/ml, respectively. Here, it was demonstrated that organic acids can be used for pretreatment of lignocellulosic materials to enhance of hydrolysis process, which could be integrated to other applications for various biorefinery processes.

***Keywords*—**Lignocellolusic biomass, pretreatment, organic acid response surface methodology, biorefinery.

I. Introduction

DUE to the increasing of energy consumption and uncertainty of fuel supply, we need to find an alternative energy to substitute the use of fossil fuel. Additionally, combustion of fossil fuels releases CO_2, SO_X and NO_X, which are the major pollutants of the environment and cause greenhouse effect [1]. Biofuel is an alternative energy that currently gained high attention in research and industrial sections. Thus, exploration of raw materials for biofuel production is also considered as a significant issue.

Lignocellulosic materials include agriculture residues, forest residues and dedicated energy crops, for example corn stover, sugar bagasse, switch grass, and rice straw. Lignocelluloses consist of three types of polymers: cellulose, hemicellulose, and lignin [2]. Both cellulose and hemicellulose could be hydrolyzed to monosaccharide or simple sugar that, subsequently, could be fermented to biofuel. However, one of most important problem to produce biofuel from lignocelluloses is the resistance to biodegradation or hydrolysis [3]. Therefore, pretreatment is the process to alter such properties to improve enzymes and microbe degradation.

In general, pretreatment methods could be done in different ways, for example, chemical, physical, and biological [4], [5]. And each method has different mechanism and efficiency to modify the properties of lignocellulose. Previously, inorganic acid pretreatment such as sulfuric acid, hydrochloric acid, and nitric acid has been widely studied due to their high efficiency [6]-[8]; however, using these strong acids can cause corrosion to the equipments, and quite dangerous. Despite, organic acid pretreatment was investigated as an alternative option, its efficiency was shown to be relatively less [9], [10]. Still, the advantage of using organic acid pretreatment is that it has lower amounts of inhibitory by-products than inorganic acid pre-treatment [11].

Here, this study is mainly focuses on the application of organic acid pretreatment on rice straw to enhance the hydrolysis efficiency by commercial cellulase enzyme. To find the optimal condition for organic acid pretreatment, the Response Surface Methodology (RSM) is applied to generate mathematic model that explains the correlations of pretreatment parameters and sugar yields from hydrolysis. The statistical analysis of this study could be used to select the candidate of organic acid for further pretreatment research and readily to be applied in up-scale process.

II. Materials and Methods

A. Preparation and Analysis of Rice Straw

Rice straw collected from a local farm in Ayuthdhaya province, Thailand was dried in hot air oven at 80^{o}C until constant weight was achieved. The rice straw was chopped to 2–3 cm-long pieces. Contents of cellulose, hemicelluloses and lignin of rice straw were determined using the method described by [12]. The total solids (TS) and volatile solids (VS) were tested according to the standard methods [13]. The contents of cellulose, hemicellulose, and lignin in rice straw were 36.06%, 28.22%, and 12.63% respectively. The total solids (TS) and volatile solids (VS) content of rice straw was 92.35% and 85.96%.

B. Experimental Design

Design-Expert software (version 7.0.0 Stat-Ease, USA) was used to generate the RSM experimental design, mathematic model, and calculate optimized condition. Box-Behnken

R. Hengaroonprasan and K. Cheenkachorn (corresponding author) are with Department of Chemical Engineering, King Mongkut's University of Technology North Bangkok (KMUTNB), Bangkok, 10800 Thailand (e-mail:rotchanaphan_h@hotmail.com, kraipat@gmail.com).

M. Sriariyanun is with The Sirindhorn International Thai-German Graduate School of Engineering, KMUTNB, Bangkok, 10800 Thailand (e-mail: macintous@gmail.com).

P. Tantayothai is with Department of Biology, Srinakharinwirot University, Bangkok, 10110 Thailand (e-mail: prapakorn@g.swu.ac.th).

S. Roddecha is with Department of Chemical Engineering, Kasetsart University. Bangkok, 10900 Thailand (e-mail: supacharee@gmail.com).

Design was applied to reduce the numbers of experiments, with a total of 17 runs. The parameters of pretreatments or independent variables that were selected to be studied here included reaction time (X_1), pretreatment temperature (X_2), and acid concentration (X_3). And the dependent variable was sugar yield (Y). The pretreatment conditions were organic acid concentrations of 5–15% (w/w) or inorganic acid concentrations of 0.5–2% (w/w), temperatures of 100–140°C, and reaction times of 30–60 min. The coded value levels of the independent variables, including max = +1, mid = 0, min = -1, were selected for the optimization as shown in Table I. The experimental data were analyzed by using statistical software to accommodate the second-order polynomial regression model:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j$$

TABLE I
EXPERIMENTAL DESIGN TO TEST THE EFFECTS OF INDEPENDENT VARIABLES (TEMPERATURE (X_1), TIME (X_2) AND CONCENTRATION (X_3)) ON BIOGAS YIELD (Y))

Std	Run	X_1	X_2	X_3	Sugar yield 72 hour (mg/ml)			
					HCl	Oxalic acid	Acetic acid	Citric acid
17	1	0	1	-1	7.4581	9.1870	6.8253	6.2264
2	2	1	-1	0	6.8818	10.2492	6.9722	5.9100
5	3	0	-1	-1	7.8310	11.4244	6.3394	5.7744
13	4	0	-1	1	6.7688	9.9102	6.9609	6.2264
11	5	-1	1	0	5.7857	7.8988	6.8705	6.2038
8	6	0	0	0	7.8423	8.3282	7.1417	5.6275
15	7	0	1	1	8.5542	10.4413	7.2321	6.6219
10	8	1	0	-1	8.1700	9.6503	6.9722	6.0456
9	9	0	0	0	8.1474	8.1813	7.1191	5.5710
7	10	0	0	0	8.0796	8.3282	7.0174	5.6388
4	11	-1	-1	0	8.3169	8.3508	6.3846	6.3733
6	12	-1	0	1	6.7236	8.1248	6.4072	5.5823
1	13	-1	0	-1	5.9552	7.7858	6.6897	5.9552
12	14	0	0	0	7.7745	8.1135	7.1078	5.6162
14	15	1	1	0	7.4807	9.7746	6.9722	6.5202
16	16	1	0	1	7.3564	8.9158	6.7688	6.5202
3	17	0	0	0	8.2039	8.2265	7.0852	5.6388

C. Organic Acid Pretreatment

The experiment consists of 3 types of organic acids including acetic acid ($C_2H_4O_2$), citric acid ($C_2H_2O_4$) and oxalic acid ($C_6H_8O_7$). Additionally, hydrochloric acid (HCl) was also tested as a reference. All pretreatments were performed in a screw-capped bottle with a total volume of 50 ml and 10% (w/w) of rice straw loading in acid solution. The samples were then heated in a hot air oven with the set parameters of temperature and time based on RSM experimental design. After pretreatment, solid residue was separated by filtration using fritted-glass filter and washed with deionized water three times. Samples were dried at 60 °C until constant weight was achieved, and kept in the sealed bags until used in subsequent enzymatic hydrolysis.

D. Enzymatic Hydrolysis

Pretreatment efficiency of each experimental runs was assessed based on digestibility of the pretreated solid residues using a commercial cellulase enzyme mixture (containing 175 µl of Celluclast 1.5 L (20 FPU) (Sigma, USA, produced by *Trichoderma reesei* ATCC 26921) and 125 µl of Novozyme 188 (Sigma, USA, Cellobiase produced by *Aspergillus niger*)). Each enzymatic hydrolysis experiment was conducted in a screw-capped plastic tube containing 50 mM sodium citrate buffer (pH 4.7) with volume 20 ml and a solid loading of 0.5 g. The reactions were incubated at 45°C, 200 rpm for 72 h in a shaking incubator. The amount of liberated reducing sugars of hydrolysate was determined by using the 3,5-dinitrosalisylic acid (DNS) method [14].

III. RESULTS

For RSM optimization experiment, the effects of three independent variables on sugar yield (Y) were studied including temperature (X_1), treatment time (X_2) and acid concentration (X_3) based on the design (Table I). The coded value levels of the independent variables including max = +1, mid = 0 and min = -1 were selected for the optimization. The analysis of variance (ANOVA) was used to evaluate empirical relationship between the response and independent variables and expressed by a second-order polynomial equation. Additional variables included here are the two parameter interactions of (X_1X_2), (X_2X_3) and (X_1X_3) and the second order effects of (X_1^2), (X_2^2) and (X_3^2). The final mathematical models of dilute inorganic acid pretreatments associated to the responses in terms of coded factors (1)-(4) and actual factors (5)-(8) of Hydrochloric acid, Acetic acid, Citric acid, and oxalic acid, respectively, were shown below:

$$\text{Sugar} = 7.85+(0.39X_1)-(0.065X_2)-(1.412x10^{-3}X_3) +(0.78\,X_1X_2)+(0.54\,X_2X_3)-(0.77\,X_1^2) \quad (1)$$

$$\text{Sugar} = 7.06+(0.17X_1)+(0.16X_2)+(0.068X_3) - (0.22X_1^2 -0.18\,X_3^2) \quad (2)$$

$$\text{Sugar} = 5.80+(0.16\,X_2)+(0.43\,X_2^2) \quad (3)$$

$$\text{Sugar} = 8.24+(0.80X_1)-(0.33X_2)-(0.082X_3)-(0.27X_1X_3) +(0.69X_2X_3)-(0.39X_1^2)+(1.23X_2^2)+(0.78\,X_3^2) \quad (4)$$

$$\text{Sugar} = 5.12178+(0.36242X_1)-(0.37729X_2)-(2.16018X_3) +(2.60842x10^{-3}X_1X_2)+(0.04796X_2X_3)-(1.91825x10^{-3}X_1^2) \quad (5)$$

$$\text{Sugar} = 3.04332+(0.13822X_1)+(0.010358X_2)+(0.15511X_3) -(5.41211x10^{-4}X_1^2)-(7.07737x10^{-3}X_3^2) \quad (6)$$

$$\text{Sugar} = 9.20929-(0.16228X_2)+(1.9224x10^{-3}X_2^2) \quad (7)$$

$$\text{Sugar} = 5.45602+(0.30365X_1)-(0.60510X_2)-(0.73201X_3) -(2.68375x10^{-3}X_1X_3) +(9.22833x10^{-3}X_2X_3) -(9.85925x10^{-4}X_1^2)+(5.45413x10^{-3}X_2^2)+(0.031120X_3^2) \quad (8)$$

The response surface plots were used to analyze the individual and interactive effects of the independent variables on the responses and were used to determine the optimum conditions for each variable in the rice straw decomposition process. The significance of these experiments was analyzed by ANOVA and expressed in the form of the F-value (Table II). Quadratic regression model is suggested to evaluate the experimental data of all experimental sets with coefficient of determination (R-squared) value of 0.7763, 0.8195, 0.8780 and 0.9852 in hydrochloric, acetic acid, citric acid and oxalic acid experimental set, respectively, which advocated the significance of the model.

Furthermore, the values of "Prob. > F" less than 0.05 and F-value implies the model are significant. The cut-off criteria of statistically significance with Prob. > F with less than 0.1 was applied to each model term. The most significant model terms: (X_1X_2 and X_1^2) are terms of hydrochloric pretreatment, (X_1, X_2 and X_1^2) are terms of acetic acid pretreatment. X_2^2 is citric acid pretreatment terms and (X_1, X_2, X_1 X_3, X_2 X_3, X_1^2, X_2^2 and X_3^2) are significant terms of oxalic acid pretreatment.

Mathematic model derived from experimental data of the 4 types of acid pretreatments can be used to find the optimal condition of each pre-treatment. In hydrochloric acid set, the optimal conditions were 1.97% concentration and incubated at 133.16°C for 59.93 min. In acetic acid set, the optimal conditions were 11.24% concentration and incubated at 127.76°C for 58.73 min. In citric acid set, the optimal conditions were 9.05% concentration and incubated at 117.26°C for 60 min. In oxalic acid set, the optimal conditions were 5.04% concentration and incubated at 137.11°C for 30.01 min. The maximum sugar yields are 8.72 mg/ml, 7.24 mg/ml, 6.39 mg/ml and 11.90 mg/ml, respectively. In addition, after the hydrolysis process, the cellulose, hemicellulose, and lignin contents of solid residues were analyzed (Table III). The result showed that acetic acid and oxalic acid have the better ability to eliminate the lignin compared to the untreated rice straw and hydrochloric acid. Especially, oxalic acid has the highest ability to reduce lignin content with 76.92 percentages compared with non-pretreated rice straw. Altogether, oxalic acid pretreatment was suggested to be the best candidate for rice straw pretreatment.

Response surface plot is used to demonstrate the effect and interaction of the variables, which will be displayed in the form of two independence variables at a time. In Fig. 1, the effect of the independent variables on the sugar yields of oxalic acid pretreatment was shown. Clearly, the interacting effects of two parameters were observed. For example, between the acid concentration and temperature, the sugar yield was substantially increased when the temperature was rising, at low acid concentration. However, the effect of temperature is subtle at high acid concentration. This observation support the importance of respond surface plot that help us to better understand the interacting effects of two parameters.

To validate the response surface mathematic model, the pretreatment on rice straw by organic acids were performed again with predicted optimal condition. After pretreatment, solid residues of rice straw were subjected into enzymatic hydrolysis and the amount of sugar was determined to indicated the degree of rice straw decomposition. Sugar contents in hydrolysates were measured at 2, 4, 6, 12, 24, 48 and 72 hour as shown in Fig. 2. The results indicated that degradation of rice straw increased with time and all acid pretreatment enhanced the hydrolysis efficiency compared to the control, unpretreated rice straw. Pretreatment of rice straw with oxalic acid provided highest sugar yield (10.67 mg/ml), followed by hydrochloric acid, acetic acid and citric acid showed that the contents of released sugars in hydrolysis were close to the predicted amounts from the model. For example, citric acid, the sugars obtained from model and validated in experiments are 6.39 and 6.36 mg/ml, respectively, which is only 0.46% different (Table III).

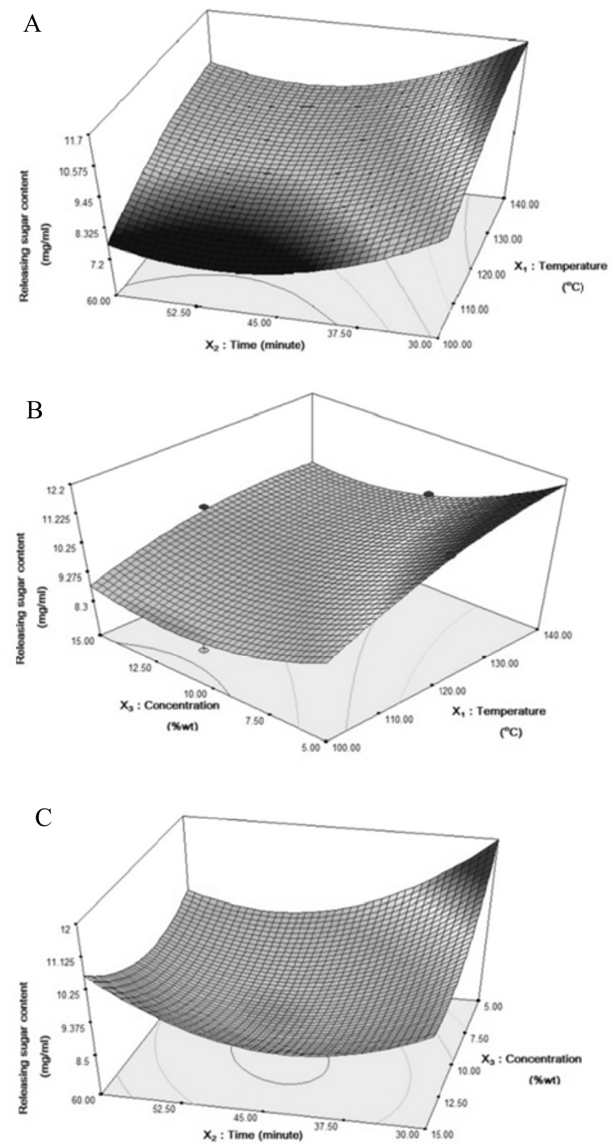

Fig. 1 Response surface plots of oxalic acid pretreatment represent effects of the interaction between the independent variables on the sugar yields. (A) Interaction between temperature and time; (B) interaction between temperature and concentration; (C) interaction between temperature and time

TABLE II
RESULTS FROM REGRESSION ANALYSIS OF THE DESIGN

Source	HCl			Acetic acid			Citric acid			Oxalic acid		
	Mean square	F-value	Prob>F	Mean square	F-value	Prob>F	Mean square	F-value	Prob>F	Mean square	F-value	Prob>F
model	1.22	3.71	0.03	0.16	4.35	0.02	0.50	5.93	0.01	2.25	66.57	< 0.0001
X_1	1.21	3.66	0.08	0.22	6.03	0.03	-	-	-	5.17	153.15	< 0.0001
X_2	0.03	0.10	0.76	0.19	5.24	0.04	0.21	2.46	0.14	0.87	25.68	0.001
X_3	0.00002	0.00005	0.99	0.04	1.00	0.34	-	-	-	0.05	1.59	0.24
X_1X_2	2.45	7.43	0.02	-	-	-	-	-	-	0.29	8.54	0.02
X_1X_3	1.16	3.53	0.09	-	-	-	-	-	-	1.92	56.79	< 0.0001
X_1^2	2.49	7.56	0.02	0.20	5.37	0.04	-	-	-	0.65	19.41	0.002
X_2^2	-	-	-	-	-	-	0.79	9.40	0.01	6.34	187.92	< 0.0001
X_3^2	-	-	-	0.13	3.58	0.08	-	-	-	2.55	75.53	< 0.0001

TABLE III
THE RESULTS OF DILUTE ACID PRETREATMENT UNDER OPTIMAL CONDITIION

Method	Liquid phase (hydrolysate)			%mass loss	Solid phase		
	Predict (mg/ml)	Actual (mg/ml)	%conversion		Cellulose g/g Rice straw	Hemicellulose g/g Rice straw	Lignin g/g Rice straw
non-pretreat	-	3.98	13.41	18.21	0.27	0.27	0.13
Acetic acid	7.24	7.07	23.81	30.89	0.28	0.26	0.08
Citric acid	6.39	6.36	21.42	36.42	0.22	0.19	0.10
Oxalic acid	11.90	10.67	35.95	39.87	0.23	0.21	0.03
Hydrochloric acid	8.72	8.53	28.74	35.91	0.22	0.19	0.10

Ultimately, the fiber analysis of remaining rice straw solid residue after hydrolysis showed that oxalic acid pretreatment caused the biggest loss of rice straw mass supporting the result of highest sugar conversion. Additionally, the remaining lignin content in the oxalic acid pretreated sample was the least suggesting its benefit to remove inhibitor of hydrolysis reaction.

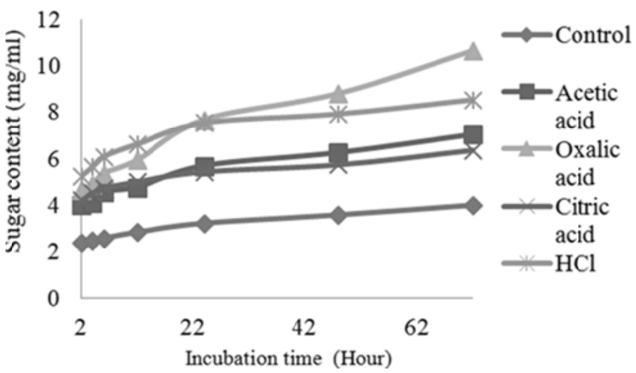

Fig. 2 Sugar contents of organic acids and inorganic acid pretreated samples at 2, 4, 6 12, 24, 48 and 72 hours

IV. DISCUSSION

Based on results of pretreatment experiments, organic acids were demonstrated their applications on enhancement of hydrolysis efficiency of cellulase enzyme. Comparing to unpretreated rice straw, sugar contents obtained from organic acid pretreated samples has more than 2 times. Especially, oxalic acid pretreatment has higher efficiency than hydrochloric acid. Finding the optimum conditions for pretreatment of rice straw by using organic acids based on RSM, the results indicate that optimization of this empirical case can be useful in the other applications to provide sugars from lignocellulosic biomass for biofuel production or biorefinery via fermentation process in the future.

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