PROCESS OPTIMIZATION FOR SUGARS PRODUCTION FROM RICE STRAW VIA PRETREATMENT WITH SULFUR TRIOXIDE MICRO-THERMAL EXPLOSION

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The effects of sulfur trioxide micro-thermal explosion (STEX) and enzyme loading on reducing sugars conversion of STEX-treated rice straw and enzymatic hydrolysates were researched. Important process parameters in the pretreatment of biomass were identified by a Plackett-Burman design, and parameters with significant effects were optimized using a Box-Behnken design (BBD) and response surface methodology (RSM). The optimal conditions were a temperature of 80 °C and a treatment time of 30 min when only single factors were considered. Meanwhile, glucose and xylose were primary components in the enzymatic hydrolysates. Subsequently, STEX time, liquid-solid ratio, and soaking temperature were the main factors governing the enzymatic saccharification of rice straw. The optimum pretreatment conditions were STEX time 23.3 min, liquid-solid ratio 13.3 (V/m), and soaking temperature 62.2 °C. The chemical composition analysis of straw further demonstrated that STEX collaborative dilute lye pretreatment could remove lignin and hemicellulose.

Keywords: Rice straw; Sulfur trioxide micro-thermal explosion (STEX); Dilute lye; Saccharification; Response surface methodology

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INTRODUCTION

In order to utilize rice straw and other abundant agriculture residues for cellulosic ethanol, a variety of different pretreatment methods to change the lignocellulosic structure have been reported and used, such as milling (Sato *et al.* 2009; Zhu *et al.* 2009), dilute acid (Taherzadeh and Karimi 2007), steam explosion (Mukhopadhyay and Fangueiro 2009), liquid hot water, dilute alkali, wet oxidation, and ammonia fiber explosion (AFEX) (Kumar and Wyman 2009; Taherzadeh and Karimi 2008; Das and Chakraborty 2009; Goswami *et al.* 2009; Li *et al.* 2009; Liu and Wyman 2005). The general purpose of these methods is to remove or alter the hemicellulose or lignin, decrease the crystallinity of cellulose, and increase the enzymatic hydrolysis area (Goering and Van Soest 1970; Mosier *et al.* 2005; Zhao *et al.* 2009). Presently, most of these pretreatment methods require high-temperature or high-pressure conditions, and the application of chemicals may be toxic to the enzymes or the fermentative micro-organisms. However, the removal of these toxicants is always costly and complicated.

A new approach, namely sulfur trioxide micro-thermal explosion (STEX) collaborative dilute alkali lye pretreatment, has been proposed, which can destroy the straw structure under ambient pressure (Yao *et al.* 2011). The resulting cellulose could be used to produce cellulose ethanol by saccharification and fermentation without any inhibition; so this innovative technology possesses a potential value for application on a large-scale. However, most of the previous studies have been conducted merely by one-variable-at-a-time experiments, which are time-consuming, and may also result in one-side conclusions for optimizing a multivariable system without consideration of the interactive effects among the variables.

Therefore, response surface methodology (RSM) was used to optimize the parameters of the STEX collaborative dilute alkali lye pretreatment in this work. RSM is a collection of statistical techniques for designing experiments, building models, and evaluating the effects of factors (Yue *et al.* 2008; Singh *et al.* 2010). It extracts maximum information with a minimum number of runs. For the purpose of screening out the main parameters that affect STEX collaborative dilute alkali pretreatment on digestion of rice straw, a Plackett-Burman design was used. Then, a Box-Behnken design (BBD) was applied in this study to optimize the selected pretreatment conditions. In addition, the changes in the main chemical components in pretreated straw were also used to evaluate the influence of STEX collaborative dilute alkali on recalcitrant structures.

EXPERIMENTAL

All experiments were carried out three times, and the given values are the mean values ± standard deviation (SD).

Materials and Microorganisms

Rice straw used in the experiments was harvested at maturity in October 2010 from a local farm in Hefei, China. It was collected and stored with adequate ventilation to dry under natural conditions. Before any pretreatment, biomass was cut to 1 to 2 cm in length and used for further treatment (Yao *et al.* 2011). The green wood mold ZY-1, which was isolated from nature, acted as the starting strain for composite mutagenesis via UV and plasma to obtain predominant strains of ZY-2.

Optimization of Parameters for Pretreatment

Optimization of parameters for pretreatment of biomass was performed in two stages. Initially, seven variables were screened using a Plakett-Burman design to identify parameters that significantly influenced pretreatment. In the second stage, the levels of these parameters were optimized using response surface design.

Screening of parameters affecting pretreatment by Plackett-Burman design

The Plackett-Burman design is a powerful and efficient mathematical approach to determine and screen out the effect of parameters. It offers a good and fast screening procedure and mathematically computes the significance of a large number of factors in one experiment (Lv *et al.* 2008; Reddy *et al.* 2008; Singh *et al.* 2010). In this study,

STEX temperature, STEX time, oleum volume, alkali concentration (NaOH), soaking temperature (in alkali lye), soaking time, and the liquid-solid ratio were selected as independent variables (Ma *et al.* 2009). These variables were investigated and 16 experiments were carried out. Each variable was set at two levels, a high level and a low level. The experimental design is given in Table 1a. The significance of regression coefficients was tested by t-test (Table 1b).

Table 1a. Plackett-Burman Design Matrix for the Screening of Variables Influencing Pretreatment of Biomass

Code	A	B	C	D	E	F	G	SR (%)
1	100.00	5.00	10.00	1.00	30.00	7.00	5.00	35.8
2	100.00	30.00	2.00	1.00	30.00	7.00	15.00	47.5
3	100.00	30.00	10.00	5.00	80.00	7.00	15.00	50.8
4	60.00	5.00	10.00	5.00	80.00	1.00	5.00	36.1
5	100.00	5.00	10.00	5.00	30.00	1.00	15.00	37.6
6	100.00	5.00	2.00	5.00	80.00	7.00	5.00	39.4
7	100.00	30.00	2.00	5.00	30.00	1.00	5.00	42.7
8	60.00	30.00	2.00	5.00	80.00	1.00	15.00	46.9
9	60.00	30.00	2.00	1.00	80.00	7.00	5.00	45.7
10	60.00	5.00	2.00	5.00	30.00	7.00	15.00	35.2
11	60.00	5.00	2.00	1.00	30.00	1.00	5.00	30.2
12	60.00	30.00	10.00	5.00	30.00	7.00	5.00	40.3
13	100.00	30.00	10.00	1.00	80.00	1.00	5.00	46.3
14	60.00	30.00	10.00	1.00	30.00	1.00	15.00	45.9
15	100.00	5.00	2.00	1.00	80.00	1.00	15.00	41.8
16	60.00	5.00	10.00	1.00	80.00	7.00	15.00	44.2

Table 1b. Actual Level of Variables Tested with the Plackett-Buraman Design and their Effect on Pretreatment of Biomass

Code	Parameter	Low Level (-1)	High Level (+1)	t Value	P Value	Confidence Level (%)
a	0	60	100	1.15	0.0059	99.41
b	STEX Time (min)	5	30	4.17	<0.0001	>99.99
c	Oleum Volume (ml)	2	10	0.54	0.1205	87.95
d	Alkali Concentration (%)	1	5	-0.46	0.1733	82.67
e	Soak Temp. (°C)	30	80	2.31	<0.0001	>99.99
f	Soak Time (h)	1	7	0.77	0.0036	99.64
g	Liquid-Solid Ratio	5	15	2.15	0.0001	99.99

Optimization of screening parameters by Box-Behnken design

A Box-Behnken factorial design with three factors and three levels, including three replicates at the centre point, was used for the optimization of pretreatment conditions. In this experiment, BBD was used to evaluate the effects of the interaction of the main factors (STEX time (A), liquid-solid ratio (B), and soaking temperature (C)) on rice straw saccharification. The experimental designs with the observed responses and predicted values for rice straw saccharification are presented in Table 2. A polynomial quadratic equation (EQ1) was fitted to evaluate the effect of each independent variable to the responses,

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC \quad (1)$$

where Y is the predicted response, β_0 is a constant, β_1, β_2, and β_3 are the linear coefficients, β_{12}, β_{23}, and β_{13} are the cross-coefficients, and β_{11}, β_{22}, β_{33} are the quadratic coefficients. The response surfaces of the variables inside the experimental domain were analyzed using Design Expert software. Subsequently, five additional confirmation experiments were conducted to verify the validity of the statistical experimental strategies.

Table 2. Box-Behnken Design Matrix for Optimization of Parameters Identified by Plackett-Burman Design

Standard Order	STEX Time (min)	Liquid Solid Ratio	Soak Temperature (°C)	Actual Saccharification Rate (%)	Predicted Saccharification Rate (%)
1	5.00	5.00	55.00	37.3	36.93
2	60.00	5.00	55.00	25.2	24.23
3	5.00	15.00	55.00	44.8	45.77
4	60.00	15.00	55.00	29.4	29.78
5	5.00	10.00	30.00	41.8	39.73
6	60.00	10.00	30.00	27.3	25.84
7	5.00	10.00	80.00	41.7	43.18
8	60.00	10.00	80.00	26.3	28.38
9	32.50	5.00	30.00	35.5	37.95
10	32.50	15.00	30.00	43.8	44.90
11	32.50	5.00	80.00	41.8	40.70
12	32.50	15.00	80.00	50.6	48.15
13	32.50	10.00	55.00	48.5	48.80
14	32.50	10.00	55.00	48.4	48.80
15	32.50	10.00	55.00	49.1	48.80
16	32.50	10.00	55.00	48.8	48.80
17	32.50	10.00	55.00	49.2	48.80

STEX Collaborative Dilute Alkali Pretreatment

Rice straw, which was cut into small pieces about 2 to 3 cm in length, was hung over the upper portion of test tube and oleum (sulfur trioxide 50%) was in the bottom of the test tube. STEX treatments were performed at the given time (from 5 min to 60 min) and a specific temperature range (from 60 °C to 100 °C). Afterwards, the mixture was soaked in the dilute lye, and then the filtrate and residue were separated by eightfold gauze filtration. After pretreatment, the pretreated solids were collected and washed extensively with deionized water until a neutral pH was reached. The collected solids were used for determination of total solids and enzymatic hydrolysis (Yao *et al.* 2011).

Enzymatic Hydrolysis

Crude cellulase from *Trichoderma viride* ZY-2 (collection in this experiment) with 3 IU/mL enzyme solution was used for hydrolysis experiments. The pretreated rice straws at 7% solids loading (grams dry weight per 100 mL) in 0.1 M citrate buffer (pH 4.8) were preincubated in flasks in a shaking water bath at 50 °C at 150 rpm for 48 h.

Analytical Methods

Cellulose, hemicellulose, lignin content, and ash content of the rice straw were estimated by the methods of Goering and Van Soest (1970) as well as Han and Rowell (1997). The total cellulase activity (filter paper activity, FPA) was assayed according to IUPAC recommendations by using filter paper as the substrate (Ghose 1987).

In the enzyme hydrolystate analysis, test equipment was mainly composed of a Shimadzu LC-6A high performance liquid chromatograph (HPLC), RID-6A differential refraction detector, and an amino chromatographic column. The detection conditions were with a mobile phase of acetonitrile + H_2O (75+25), flow velocity 1.0 mL/min, room temperature (22±2 °C), and sample size 20 μL.

The enzymatic hydrolysis reaction was monitored by withdrawing samples from the supernatant periodically and measuring the release of soluble reducing sugars by DNS assay using D-glucose as a standard (Miller 1959).

Saccharification rate (SR) of pretreated rice straw was calculated as follows,

$$SR = \frac{RG}{RS} \times 0.9 \times 100\% \quad (2)$$

where RG is the dry weight of reducing sugars in supernatant, RS is the dry-weight rice straw before pretreatment, and 0.9 is the mass ratio of anhydroglucose to free glucose.

RESULTS AND DISCUSSION

Effects of STEX Factors on Reducing Sugars Conversion of Rice Straw

Figure 1a shows the effects of STEX treatment temperature on the percent conversion of rice straw to reducing sugars, and Fig. 1b shows the effects of STEX time on conversion to reducing sugars in different enzyme concentrations.

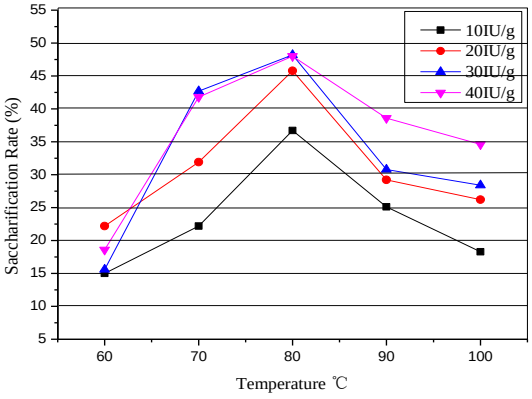


Fig. 1a. Effects of STEX treatment temperature on reducing sugars conversion of STEX-treated rice straw at 30 min in different enzyme concentrations

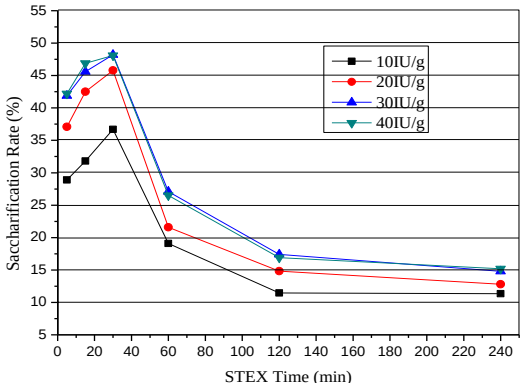


Fig. 1b. Effects of STEX treatment time on reducing sugars conversion of STEX-treated rice straw at 80 °C in different enzyme concentrations

In these figures, SO_3 loading, alkali concentration (NaOH), soaking temperature (in alkali lye), soaking time, liquid-solid ratio, and time of hydrolysis were all held constant. As Fig. 1a shows, after increasing temperature from 60 °C to 100 °C, the conversion of reducing sugars increased and then declined. Apparently, an increase in treatment temperature beyond 80 °C significantly decreases the SR. The higher temperature could reduce moisture content and decrease the efficiency of the STEX treatment. Simultaneously, the maximum conversion occurred with rice straw treated at 80 °C and an enzyme concentration of 30 IU/g of substrate. Thus 80 °C was selected as the optimal temperature in the single factor experiment.

It is apparent from Fig. 1b that reducing sugars conversion increased with increasing STEX treatment time and the maximum conversion occurred with rice straw treated at 30 min. But, when the STEX treatment time was longer than 30 min, the SR sharply declined. The cause of this result may be that beyond a certain time range, STEX treatment produced a poisonous substance for enzyme hydrolysis. In addition, a further increase in enzyme concentration beyond 20 IU/g of substrate did not have much additional benefit.

Comparing Different Enzyme Loadings and HPLC Analysis of Saccharification Liquid

The enzyme loading during saccharification also determined the rate and extent of polysaccharide hydrolysis. Under the same pretreatment conditions, the hydrolysis yield could be enhanced by using higher enzyme loading. Similarly, an effective pretreatment can reduce the required enzyme loading substantially. STEX did indeed significantly reduce required enzyme loadings, or in other words, enhanced the catalytic efficiency of enzyme. The effects of different enzyme concentrations on the hydrolysis of STEX-treated rice straw were studied. These results are summarized in Fig. 2a, and they show that by cutting enzyme levels from 40 to 20 IU/g of substrate, the reducing sugars conversion leveled off and then declined and obtained a higher conversion rate at 30 IU/g of substrate. A further cut to 10 IU/g of substrate resulted in a 12% reduction in reducing sugars conversion. Thus, the enzyme loading was set at 30 IU/g of substrate in the following experiment.

The results of HPLC analysis (Fig. 2b) illustrated that the saccharification liquid was mainly composed of monomer glucose and xylose. At the same time, there was also a small amount of arabinose, cellobiose, or other forms of sugar product. It implies that the enzymatic hydrolysates could be fermented or separated to produce chemical raw materials and have enormous economic potential.

Preliminary Results

Preliminary experiments were performed to determine the main factors and the appropriate range by Placket-Burman design. The effects of different factors (STEX temperature, STEX time, oleum volume, alkali concentration (NaOH), soaking temperature (in alkali lye), soaking time, and liquid-solid ratio) were evaluated on the basis of reducing sugar released from rice straw after the pretreatment (Table 1a). Among all the variables, STEX time (A), liquid-solid ratio (B), and soaking temperature (C) were identified as the crucial and contributing variables (as shown by Table 1b) with a range of

5 to 60 min, 5 to 15 (V/m), and 30 to 80 °C, respectively. Although the sample treated for a STEX time of 30 min could reach very high SR, the STEX time range of 5 to 60 min was used in the BBD to examine if they would improve over time. The ranges of 5 to 15 (V/m) and 30 to 80 °C were chosen as the appropriate ranges for liquid-solid ratio (B) and soaking temperature (C), in consideration of both energy efficiency and minimum water usage.

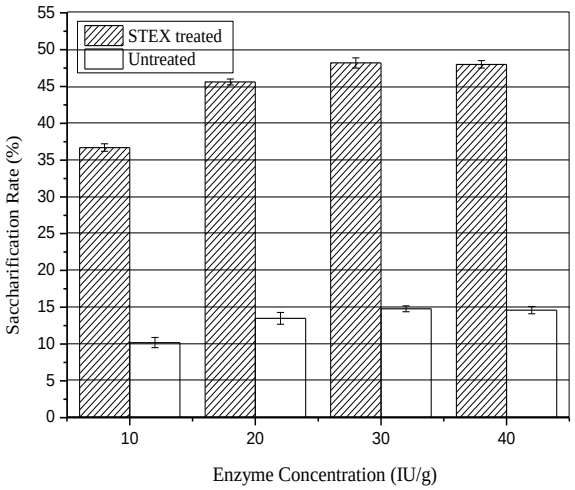


Fig. 2a. Reducing sugars conversion profile of STEX-treated rice straw at 80 and 30 min at different enzyme loadings

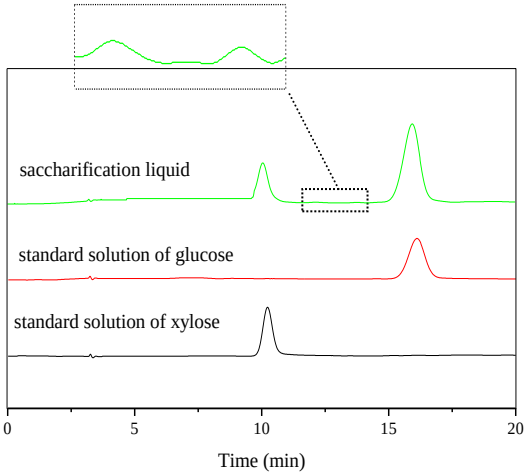


Fig. 2b. HPLC analysis of standard solution and saccharification liquid

RSM Results

Further optimizing of the effects on rice straw saccharification was achieved by employing BBD. Data were analyzed using Design Expert software to yield analysis of variance (ANOVA), regression coefficients, and a regression equation. The polynomial equation, describing the SR as a simultaneous function of the STEX time (A), liquid-solid ratio (B), and soaking temperature (C) is shown as Eq. 3.

$$Y = 2.6150 + 0.8173A + 3.2500B + 0.5688C - 0.0153A^2 - 0.1195B^2 - 0.0046C^2 - 0.0060AB - 0.0003AC + 0.0010BC \quad (3)$$

Table 3 shows the ANOVA for the fitted model. The model F value of 31.54 implies that the model was significant. At the same time, the lack-of-fit statistics, which were used to test the adequacy of the model, indicate that the P-value of 0.0050 was not significant. No abnormality was observed from the diagnoses of residuals. Thus, it can be concluded that the model was statistically sound. The P-value denoting the importance of the coefficients was also vital in understanding the pattern of the mutual interactions between the variables. The independent variables of the STEX time and the quadratic term of STEX time had notable effects on the rice straw saccharification.

Table 3. ANOVA of the Quadratic Model for the SR of the Rice Straw

Source	Sum of Squares	Degree of Freedom	Mean Square	F Value	p Value Prob > F	
Model	1219.45	9	135.49	31.54	<0.0001	significant
A	411.84	1	411.84	95.87	<0.0001	
B	103.68	1	103.68	24.14	0.0017	
C	18.00	1	18.00	4.19	0.0799	
AB	2.72	1	2.72	0.63	0.4521	
AC	0.20	1	0.20	0.047	0.8343	
BC	0.063	1	0.063	0.015	0.9074	
A2	570.24	1	570.24	132.75	<0.0001	
B2	37.58	1	37.58	8.75	0.0212	
C2	35.11	1	35.11	8.17	0.0244	
Residual	30.07	7	4.30			
Lack of fit	29.57	3	9.86	78.85	0.0050	
Pure error	0.50	4	0.12			
Corrected total	1249.52	16				

The 3D response surfaces and the 2D contour plots of the responses using Eq. 3 for the RS are shown in Fig. 3. The shapes of response surfaces and contour plots indicated the nature and extent of the interaction between different factors (Prakash *et al.* 2008). Less prominent or negligible interactions were shown by the circular nature of the contour plots, while comparatively prominent interactions were shown by the elliptical nature of the contour plots, or other types of contour plots (Fig. 3a, b, and c). Figure 3 shows that the interactive effects between A and B, B and C, as well as C and A, to a considerable degree, influenced the straw digestion. It supports previous studies from the lab that state that increasing the efficiency of biomass pretreatment could enhance hemicellulose removal and cellulose digestion (Yao *et al.* 2011). However, the SR will decline sharply when the STEX time covers a certain range. It may be due to a series of reactions that lead to a change of the enzymatic substrate that makes it not conducive to enzymatic hydrolysis. Simultaneously, with an exorbitant temperature and liquid-solid ratio, more hemicellulose and cellulose was shucked off and then leaded to reduce the quantity of availability enzymatic hydrolysis substrate, and consequently, the SR had an apparent descent.

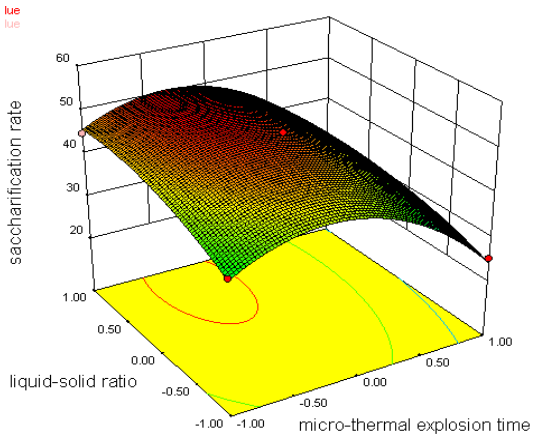
Optimization and Confirmation Experiments

The optimal conditions for STEX collaborative dilute lye pretreatment were attained by Design Expert software through a graphical optimization. Taking both cost and efficiency into account, the optimum operating parameters were found to be: A = 23.3 min, B = 13.3 (V/m), and C = 62.2 °C. Under these conditions, confirmation experiments were conducted in five replicates. The obtained mean SR was found to be largely consistent with the predicted values. Additionally, the SR of rice straw via STEX collaborative dilute lye treatment increased by 286.3%, compared to that of untreated rice straw; however, the SR of the sample treated only with lye merely increased by 93.2% (Table 4). This demonstrated the beneficial effect of the STEX collaborative dilute lye pretreatment on the enzymatic hydrolysis of rice straw. In the meantime, it further

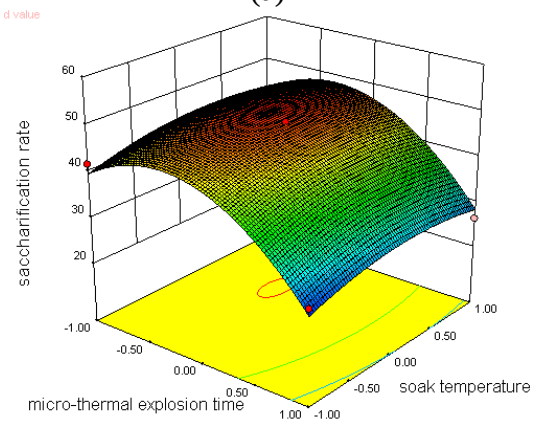
confirmed the rationality and practicability of the optimal conditions for STEX collaborative dilute lye pretreatment found in this work.

Table 4. Predicted and Experimental SR under Optimum Conditions

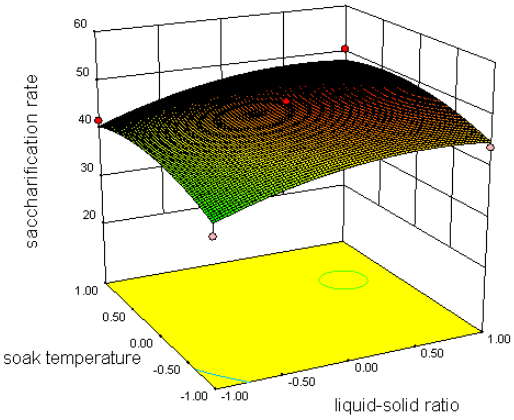
Pretreatment	Saccharification Rate (%)
Untreated	13.9 ± 0.2
Alkali treatment	27.8 ± 0.2
SO_3-alkali treatment (predicted)	53.7
SO_3-alkali treatment (measured)	54.1 ± 0.5



(a)



(b)



(c)

Fig. 3. Three-dimensional response surface and contour plots showing interactive effect between (a) STEX time (A) and liquid-solid ratio (B), (b) STEX time (A) and soak temperature(C), (c) liquid-solid ratio (B) and soak temperature (C) on rice straw saccharification

Changes of Main Chemical Components in Rice Straw with STEX Collaborative Dilute Lye Pretreatment under Optimum Conditions

All compositions were calculated based on the dry weight of samples. As shown in Table 5, the water-soluble content of the STEX-treated rice straw increased due to the content of lignin and hemicellulose. This result may be occasioned by the fact that STEX broke down the hydrogen bonds between the molecules and microporous channels within

the rice straw, which allowed for the elution of more oleoresin, small molecules, and bits of lignin/hemicellulose fragments. Simultaneously, the lignin and hemicellulose of the rice straw pretreated by STEX collaborative dilute lye decreased from 19.6% to 6.9% and 21.4% to 11.7%, respectively.

Table 5. Chemical Composition (Percent by Dry Weight) of Rice Straw

Composition (%)	Untreated straw (100.0 g)	STEX treated straw (100.8 g)	STEX assist lye treated straw (74.2 g)
Water-Soluble	14.1 ± 0.2	15.9±0.4	9.6 ± 0.5
Cellulose	39.2 ± 0.7	39.8±0.5	65.8 ± 1.0
Hemicellulose	21.4 ± 0.4	20.4±0.6	11.7 ± 0.2
Lignin	19.6 ± 0.8	18.4±0.3	6.9 ± 0.6
Ash	5.7 ± 0.1	5.5±0.4	6.0 ± 0.3

However, the cellulose content increased from 39.2% to 65.8%, which was predominantly attributed to the decrease of lignin and hemicellulose. Lignin removal could reduce binding of lignin to hemicellulose/cellulose (Han *et al.* 1997; Lu *et al.* 2002; Ahola *et al.* 2008; Ma *et al.* 2008). Meanwhile, more hemicellulose removal showed that the connection keys in the hemicellulose and cellulose were broken, thus leading to more cellulose being exposed. Above-mentioned results indicate that STEX collaborative dilute lye pretreatment could partially break the lignocellulose structure, enhance enzymatic biocatalysis, increase the yield of desired products, and recycle more cellulase. Thus, the cost associated with enzymatic saccharification of lignocellulosic biomass could be remarkably reduced.

CONCLUSIONS

1. From the above analysis, optimum conditions of the reducing sugars conversion of STEX treated rice straw were found at 80 °C, a treatment time of 30 min, and an enzyme concentration of 30 IU/g of substrate by only single factor being considered.
2. Simultaneously, glucose and xylose were the main products of enzyme hydrolysis.
3. The conditions for pretreatment of rice straw were optimized by using PBD, BBD, and RSM. The optimal conditions were found as follows: STEX time at 23.3 min, liquid-solid ratio at 13.3 (V/m), and soak temperature at 62.2 °C.
4. Under these optimum conditions, the SR increased by 286.3%. Compared to untreated rice straw, the SR of a sample treated only with lye merely increased by 93.2%. These results showed that STEX collaborative dilute lye is an efficient pretreatment method to enhance rice straw digestion and reduce enzyme dosages.
5. Chemical composition analysis further confirmed that the pretreatment could break down the lignin-hemicellulose complex, partially remove lignin and hemicellulose, and enhance enzymatic hydrolysis.

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