Optimization of Enzymatic Hydrolysis of Wheat Straw Pretreated by Alkaline Peroxide Using Response Surface Methodology

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To optimize the enzymatic conversion of widely available lignocellulosic biomass-wheat straw (WS), pretreatment facilitating the enzymatic saccharification process was first performed by alkaline peroxide, resulting in a substrate consisting of 60.17% cellulose, 29.53% hemicelluloses, and 4.59% lignin. Using response surface methodology, the combined effects of enzyme loading, substrate concentration, surfactant concentration, and reaction time on hydrolysis yield from enzymatic saccharification of WS were further investigated. The results showed that both enzyme loading and substrate concentration had interactions with surfactant concentration. A quadratic polynomial equation for predicting the hydrolysis yield was developed. The experimental results were in good agreement with predicted values. Therefore, the model could be successfully used to identify the effective combinations of the four factors for predicting hydrolysis yield.

1. Introduction

Lignocellulosic biomass such as wheat straw (WS) is an inexpensive, abundant, widely available resource. These materials, containing 75−80% of polysaccharides (cellulose and hemicellulose), can be hydrolyzed to produce monomeric sugars such as glucose and xylose, which can be further used as substrate for fermentative production of useful products.[1] Bioconversion of biomass-derived products to produce value-added fuels and chemicals offers potential economical, environmental, and strategic advantages over traditional fossil-based products.[2] Over the last decades, research efforts have been devoted to converting lignocellulosic materials to bioethanol.[3,4]

Conversion of lignocellulosic biomass to monomeric sugars can be achieved by dilute acid or cellulase. The enzymatic process is believed to be the most promising technology because enzymatic hydrolysis is milder and more specific and does not produce byproducts.[5] However, currently the bioconversion process is not economically viable because enzymatic hydrolysis is slow and requires high enzyme loading to realize reasonable rates and yields,[6] and the process is affected by many factors. These factors can be divided into two categories: structural substrate factors, e.g., degree of polymerization, degree of crystallinity, structural composition and available surface area, and enzymatic interaction, and mechanistic factors, e.g., thermal inactivation, cellulase adsorption, and synergism.[7,8] Therefore, prior to enzymatic hydrolysis, the pretreatment process, which alters the structure and compositions of the substrate, is usually employed in order to make the lignocellulosic feedstock more susceptible toward enzyme attack. The removal of lignin and hemicellulose, reduction of cellulose crystallinity, and increase of porosity in the pretreatment process can significantly improve the hydrolysis of lignocelluloses.[9] Moreover, applying surfactants has also shown promise in improving cellulose hydrolysis effectiveness.[10,11]

Intensive research demonstrates that the efficiency of the enzymatic hydrolysis of pretreated substrate depends on several process parameters such as enzyme loading, substrate concentration, reaction time, addition of surfactant, etc.[9] These factors often interact with one another; therefore, optimization of the enzymatic hydrolysis process plays an important role in improving the performance of the process. The traditional optimization method used in the enzymatic hydrolysis process, a one-factor-at-a-time technique which involves changing one independent variable (enzyme concentration, substrate concentration, reaction time, etc.) while maintaining other variables at a fixed level, not only is time consuming, laborious, and expensive but also often leads to an incomplete understanding of the system behavior, resulting in confusion and a lack of predictive ability.[12] An alternative and more efficient approach is the use of a statistical method, response surface methodology (RSM), which is an empirical modeling technique derived for evaluation of the relationship of a set of controlled experimental factors and observed results. RSM is a powerful mathematic approach for analyzing the effect of multiple variables or factors, alone or in combination, on a given process rapidly and efficiently with a minimal number of experiments while keeping a high degree of statistical significance in the results.[13] RSM can be used to optimize the enzymatic saccharification of lignocellulosic materials.

WS is one of the most abundant renewable cellulose resources worldwide consisting of 35−40% cellulose, 20−35% hemicellulose, and a relatively low content of lignin (<20%).[14,15] The low lignin content makes it an attractive hydrolysis feedstock for production of biofuels, in particular in ethanol production.[14,16] In this study, to promote enzymatic hydrolysis of WS, WS was first delignified by the alkaline peroxide method. Then, the hydrolysis of pretreated WS using cellulase was investigated in detail, with focus on the optimization of enzymatic hydrolysis of pretreated WS using RSM. Different parameters affecting hydrolysis yield of pretreated WS, including enzyme loading, substrate concentration, surfactant concentration, and reaction time, were examined. The aim of the present study was to develop a useful tool to predict and optimize the hydrolysis process of WS, which is the initial and most important stage of extensive utilization of WS using the bioconversion process.

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10.1021/ie8016863 CCC: $40.75
Published on Web 07/08/2009

Table 1. Content of Cellulose, Hemicellulose, and Lignin in WS before and after Pretreatment

	cellulose (%)	hemicellulose (%)	lignin (%)
before pretreatment	40.98	36.96	13.49
after pretreatment	60.17	29.53	4.59

2. Materials and Methods

Lignocellulosic Material and Cellulase Enzyme. WS was obtained from local farmers in Jinan, Shandong province, China. Before pretreatment with alkaline peroxide, it was cut to 1−2 cm in length, washed thoroughly with tap water to remove some sticky clay, and then dried at 60 °C in an oven (DHG-9140, Shanghai Yiheng Technology Co., Ltd., Shanghai, China). The main composition of the raw WS (dry weight basis) is shown in Table 1.

The cellulase used in this work was a commercial *Trichoderma reesei* cellulase purchased from Shanghai Kaiyang Biotech. Corp., China. Its filter paper activity was 95.70 FPU/g, and the cellobiase activity was 13.02 CBU/g.

Other chemicals (e.g., NaOH, citric acid, citric sodium, hydrogen peroxide etc.) were of analytical grade and purchased from Beijing Chemical Reagent Co., China.

Pretreatment of WS by Alkaline Peroxide. Before enzymatic hydrolysis, WS was delignified by a two-step alkaline peroxide method which has been used for postpretreating acid pretreated WS,[17] with a modified concentration of NaOH.[18] The WS was treated with 1.50% (w/v) NaOH on a shaker (150 rpm) at 50 °C for 6 h at a solid:liquid ratio of 1:25. Then, hydrogen peroxide was added to the suspensions until a concentration of 0.30% (v/v) was reached. The suspensions were kept in the dark and stirred for another 6 h. The solid residue was collected by filtration under vacuum with a 400 mesh filter cloth, washed thoroughly with tap water until the filtrate was neutral pH, and then dried at 60 °C in an oven (DHG-9140, Shanghai Yiheng Technology Co., Ltd., Shanghai, China) until a constant weight was obtained for subsequent enzymatic hydrolysis. The chemical composition of pretreated WS is shown in Table 1.

Enzymatic Hydrolysis. Hydrolysis experiments were performed in 50 mL stoppered conical flasks containing pretreated WS, cellulase enzyme powder, and 20 mL of 0.05 M citric acid/citric sodium buffer (pH 4.8), which was supplemented with 0.01% (w/v) sodium azide to prevent microbial contamination. Tween 80 (Analytical grade, Shantou Xilong Chemical Factory, Guangdong, China), a surfactant which can enhance the enzymatic conversion of lignocellulosic biomass and does not inhibit cell growth in the downstream fermentation process,[10,11] was used in these hydrolysis experiments. The flasks were incubated at 50 °C in a rotary shaker (THZ-C, Taicang Experimental Equipment Factory, Jiangsu, China) at 150 rpm. Samples (2 mL) were taken from the reaction mixture at different times according to the experimental design. Each sample taken from the hydrolysis solution was heated to 100 °C immediately for 3 min to denature the enzymes, cooled to room temperature, and then centrifuged for 20 min at 4000 rpm. The supernatant was used for reducing sugar analysis.

Analytical Methods. The content of cellulose, hemicellulose, and lignin in raw dried WS was estimated according to the methods described by Goering and Van Soest.[19] Filter paper activity and cellobiase activity were determined according to standard procedures recommended by International Union of Pure and Applied Chemistry (IUPAC).[20] One unit of filter paper activity (FPU) is defined as the amount of enzyme that forms 1 μmol of glucose (reducing sugar as glucose) per minute under the assay conditions. One unit of cellobiase activity (CBU) is the amount of enzyme that forms 2 μmol of glucose per minute from cellobiose. The reducing sugar was determined using the 3,5-dinitrosalicylic acid (DNS) method.[21] The glucose content in the hydrolysate was determined by a biosensor analyzer SBA-40 (Institute of Biology, Shandong Academy of Sciences, China). The yield of enzymatic hydrolysis was calculated as follows:[22,23]

$$\text{hydrolysis yield (\%)} = \frac{\text{reducing sugar (g)} \times 0.9}{\text{polysaccharides (hemicellulose + cellulose, g)}} \times 100$$

Infrared spectra were obtained using a Fourier transform infrared (FT-IR) spectrophotometer JASCO FT-IR-660 Plus (JASCO, Tokyo, Japan). The pretreated solid residues for FT-IR analysis were formed into a disk with KBr (3 mg in 150 mg of KBr). In scanning electron microscopy (SEM) imaging, the untreated and pretreated WS samples were sputtered with gold−palladium for 100 s, and then the coated samples were observed with a scanning electron microscope JSM-6700F (JEOL, Tokyo, Japan).

Box−Behnken Design (BBD). Box−Behnken design (BBD) is a class of rotatable or nearly rotatable second-order designs based on three-level incomplete factorial designs. The number of experiments (N) required for the development of BBD is defined as $N = 2k(k - 1) + C_0$, where k is the number of factors and C_0 is the number of central points. Basically this optimization method consists of the following steps: performing the statistically designed experiment, estimating the regression coefficients in a mathematical model, predicting the response, and checking the adequacy of the model. BBD has been applied for optimization of several chemical, physical, and biological processes.[24,25]

In the present study, the three-level four-factorial BBD is applied to investigate and validate process parameters affecting the sugar yield from the enzymatic saccharification of WS. Cellulase enzyme loading (10.00−50.00 FPU/g substrate), substrate concentration (20.00−80.00 g/L), surfactant (Tween 80) concentration (0−8.00 g/L), and hydrolysis time (24−72 h) are variable input parameters; sugar yield is an output parameter. The ranges of the above four factors examined were based on previous reports.[5,22,26] The factor levels were coded as −1 (low), 0 (central point or middle), and 1 (high). Table 2 shows the experimental parameters and experimental BBD levels used. The relation between the coded values and actual values is described according to the following equation

$$x_i = (X_i - X_i^0)/\Delta X_i$$

where x_i is the coded value of the ith independent variable, X_i is the actual value of the ith independent variable, X_i^0 is the actual value of the ith independent variable at the center point, and ΔX_i is the step change value.

In a system or process including four significant independent variables X_1, X_2, X_3, and X_4, like enzyme loading, substrate concentration, surfactant concentration, and hydrolysis time in

Table 2. Coded Values of the Variables for the Box−Behnken Design

variable	coded symbol	actual values of coded levels −1	0	1
enzyme loading (FPU/g substrate)	X_1	10.00	30.00	50.00
substrate concentration (g/L)	X_2	20.00	50.00	80.00
surfactant concentration (g/L)	X_3	0	4.00	8.00
hydrolysis time (h)	X_4	24	48	72

Table 3. Box−Behnken Design Matrix for the Four Independent Variables on the Sugar Yield in Coded Values and Experimental Results

	variables				response
run no.	X_1	X_2	X_3	X_4	Y
1	1	0	1	0	90.07
2	0	0	−1	−1	74.59
3	−1	0	−1	0	64.70
4	1	−1	0	0	95.70
5	0	1	−1	0	61.62
6	0	0	0	0	80.52
7	0	−1	−1	0	83.35
8	−1	0	0	−1	63.72
9	0	0	1	1	79.86
10	1	0	−1	0	81.83
11	0	−1	1	0	90.94
12	0	0	0	0	79.63
13	0	0	1	−1	78.21
14	1	1	0	0	76.68
15	−1	0	0	1	68.33
16	0	0	0	0	79.86
17	0	0	0	0	79.20
18	0	0	−1	1	80.85
19	0	1	0	1	66.21
20	0	1	1	0	60.23
21	1	0	0	−1	82.82
22	0	1	0	−1	67.21
23	1	0	0	1	89.74
24	−1	−1	0	0	72.65
25	−1	0	1	0	62.73
26	−1	1	0	0	53.62
27	0	−1	0	1	91.59
28	0	−1	0	−1	90.35
29	0	0	0	0	78.87

this study, the mathematical relationship between the response of these variables and the independent variables can be presented by second-degree quadratic polynomial equation

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{44}X_4^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{14}X_1X_4 + b_{23}X_2X_3 + b_{24}X_2X_4 + b_{34}X_3X_4 \quad (1)$$

where Y is the predicted value, b_0 is the constant, X_1 is the enzyme loading, X_2 is the substrate concentration, X_3 is surfactant concentration, and X_4 is the hydrolysis time, b_0 is the offset term, b_1, b_2, b_3, and b_4 are linear coefficients, b_{12}, b_{13}, b_{14}, b_{23}, b_{24}, and b_{34} are cross-product coefficients, and b_{11}, b_{22}, b_{33}, and b_{44} are quadratic coefficients. The accuracy and general ability of the above polynomial model could be evaluated by the coefficient of determination R^2 and the adjusted R^2.

For BBD with four factors ($K = 4$) and five central points ($C_0 = 5$), a total of 29 runs of experiments ($N = 29$) were required. The BBD matrix (shown in Table 3) and experimental data analysis were performed using statistical software package, Design-Expert (Version 7.1.3, 2007; Stat-Ease, Minneapolis, MN) for determining the regression coefficients of the second-order multiple regression model. The statistical significance of the model coefficients was determined by the analysis of the variance (ANOVA) combined with the application of Fisher's F test. The fitted polynomial equation was then expressed in the form of a three-dimensional surface plot using software Design-Expert in order to illustrate the relationship between the responses and the experimental levels of each of the variables examined in this study.

3. Results and Discussion

Pretreatment of WS by Alkaline Peroxide. WS was analyzed for chemical components after pretreatment by alkaline peroxide. The cellulose, hemicellulose, and lignin content of WS before and after pretreatment are shown in Table 1. As can be seen in Table 1, the percent cellulose content of pretreated WS increased to 60.17% from 40.98% of raw WS. In contrast, the percent hemicellulose and lignin content after alkaline peroxide pretreatment decreased to 29.53% and 4.59% from 36.96% and 13.49%, respectively, indicating that 65.97% lignin and 20.10% hemicelluloses of raw materials could be removed after alkaline peroxide pretreatment. Silverstein et al. performed hydrogen peroxide pretreatment of cotton stalk (2% (w/v) H_2O_2, at 121 °C for 30 min) without pH adjustment and obtained a 29.51% delignification and 30.56% solubilization of xylan.[27] Selig et al. conducted the pretreatment of corn stover with hydrogen peroxide (30% (w/w) H_2O_2, at 50 °C for 3 h) at alkaline conditions (pH 11.5) and found a loss of 56.3% for lignin and about 14.0% for hemicelluloses.[28] Sun et al. delignified rye straw with alkaline peroxide (2% H_2O_2 at pH 11.5 and 50 °C for 12 h) and showed a dissolution of 83.1% of original lignin and 70.0% of original hemicelluloses.[29] Patel and Bhatt optimized alkaline peroxide pretreatment for the delignification of rice straw and obtained 62% and 50% solubilization of lignin and hemicelluloses of raw WS, respectively.[18] Generally, an increase in cellulose content in pretreated WS would increase the level of fermentable glucose obtained from enzymatic saccharification, while a decrease in hemicellulose and lignin content could improve the efficiency of enzymatic hydrolysis of lignocellulosic materials.[30,31]

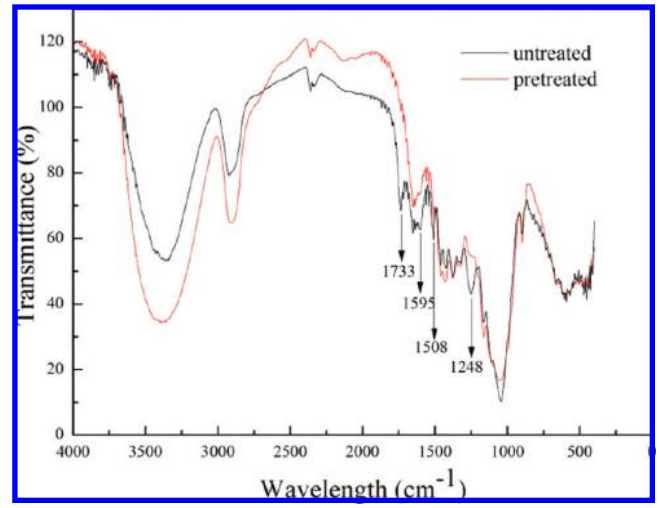

Figure 1. FT-IR spectra of alkaline peroxide pretreated WS.

The FT-IR spectrum of pretreated WS is shown in Figure 1. It is shown that the intensity of a peculiar hemicelluloses band at 1733 cm^{-1} [32,33] in the FTIR spectrum was decreased in the alkaline peroxide pretreated WS, indicating that hemicelluloses were partly removed during the pretreatment process. Sun and Tomkinson reported that the sharp peak at 1049 cm^{-1} originated from typical absorbance of xylan;[34] the reduced intensity of the peak also indicated the partial removal of hemicelluloses. Meanwhile, the peculiar absorbances of lignin at 1248[33] and 1595 cm^{-1},[35] associated with an aromatic ring stretch that is strongly associated with the aromatic C−O stretching mode, and 1508 cm^{-1} [36] are indicators of lignin. The decrease in the intensity of these peaks indicates the partial removal of lignin in the WS pretreated by the used method.

The chemical treatments can lead to structural as well as chemical changes on the fiber surfaces. SEM pictures of the WS were taken to investigate the structure of these fibers and are shown in Figure 2. Through pretreatment by alkaline

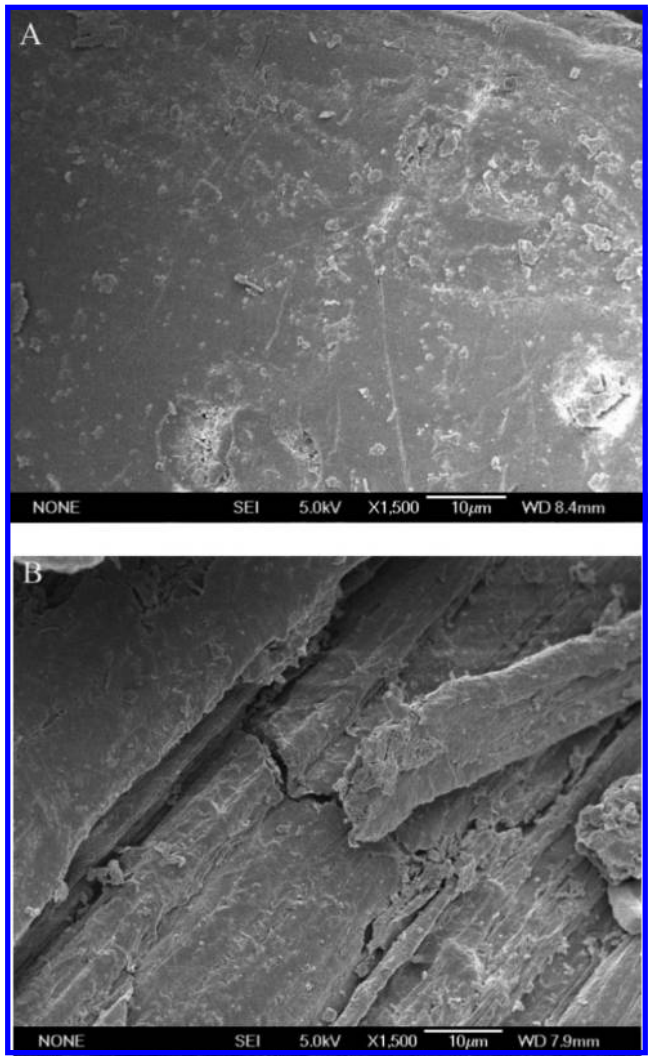

Figure 2. SEM images of WS: (A) raw WS without pretreatment; (B) WS pretreated by alkaline peroxide.

peroxide, the straw material was broken into some large fragments and appeared in porous structure (Figure 2A and 2B). The untreated sample showed an even and smooth flat surface, indicating a rigid and highly ordered surface structure (Figure 2A), while the pretreated sample had a rugged, rough, and broken surface (Figure 2B). The fragments were separated from the initial connected structure and fully exposed, thus increasing the external surface area and porosity. This would obviously favor the enzymes contacting the inner linkage, hence accelerating the biodegradation process.

Regression Models of Response. The statistical treatment combinations of the test variables along with the measured response values, expressed as hydrolysis yield corresponding to each combination, are summarized in Table 2. The application of RSM yielded the following regression equation, which was an empirical relationship between hydrolysis yield and the test variables in coded units:

$$Y = 79.60 + 10.93X_1 - 11.32X_2 + 1.26X_3 + 1.92X_4 - 3.26X_1^2 - 1.92X_2^2 - 2.47X_3^2 + 1.02X_4^2 + 0.0001X_1X_2 + 2.55X_1X_3 + 0.58X_1X_4 - 2.25X_2X_3 + 0.29X_2X_4 - 1.15X_3X_4 \quad (2)$$

The statistical significance of the above equation was checked by the F test, and the analysis of variance (ANOVA) for the

Table 4. Analysis of Variance (ANOVA) for the Quadratic Model[a]

source	SS	DF	MS	F value	probability (P) > F
model	3272.42	14	233.74	53.51	<0.0001
residual (error)	61.16	14	4.37		
lack of fit	59.56	10	5.96	14.48	
pure error	1.60	4	0.40		
total	3333.58	28			

[a] Coefficient of determination (R^2) = 0.98; ddjusted R^2 = 0.96; voefficient of variation (CV) = 2.72%; SS, sum of squares; DF, degree of freedom; MS, mean square.

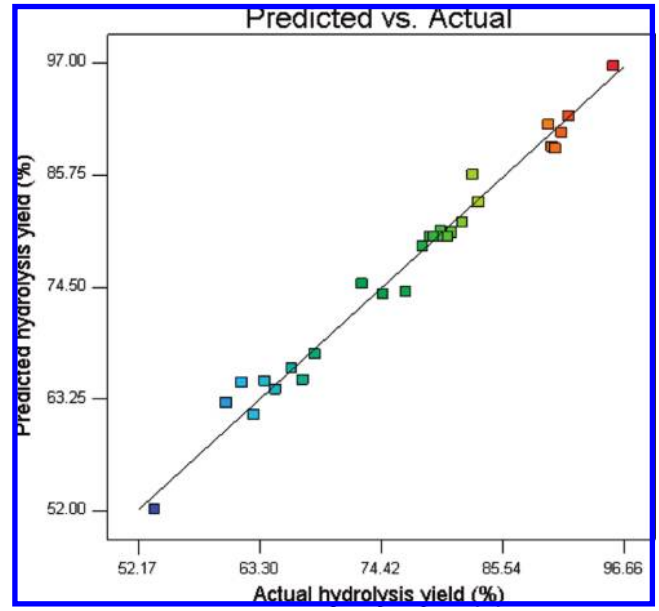

Figure 3. Observed sugar yield vs the predicted sugar yield.

response surface quadratic model is shown in Table 4. The model F value of 53.51 and values of probability (P) > F (<0.0001) showed that the model terms are significant. The coefficient of variation (CV) indicates the degree of precision with which the treatments were compared. A relatively lower value of CV (2.72%) indicated a better precision and reliability of the experiments.[37] The coefficient of determination (R^2) was calculated for 0.98 for sugar yield (see Table 4), indicating that the statistical model can explain 98% of the variability in the response and only about 1.83% of the total variation can not be attributed to the independent variables. The R^2 value is always between 0 and 1. The closer the R^2 is to 1.0, the stronger the model and the better it predicts the response. Normally, a regression model with an R^2 higher than 0.90 is considered to have a very high correlation.[38] Figure 3 shows the observed sugar yield (the response) versus those from the empirical model. As can be seen from the figure, the predicted data of the response from the empirical model agreed well with the observed ones in the range of the operating variables. The adjusted R^2 value corrects the R^2 value for the sample size and for the number of terms. A high value of adjusted determination coefficient (adjusted R^2 = 0.96) advocates for a high significance of the model. If there are many terms in the model and the sample size is not very large, the adjusted R^2 may be noticeably smaller than R^2.

Table 5 shows the F test and the corresponding P value along with the parameter estimate. The smaller the P values, the bigger the significance of the corresponding coefficient.[39] The parameter estimates and the corresponding P values suggest that, among the independent variables, X_1 (enzyme loading), X_2 (substrate loading), and X_4 (hydrolysis time) have significant

Table 5. Significance of the Coefficients of Regression

model term	parameter estimate	standard error	F value	p value
b_0	79.60	0.92		
b_1	10.93	0.60	327.90	<0.0001[a]
b_2	−11.32	0.63	327.41	<0.0001[a]
b_3	1.26	0.60	4.33	0.0562
b_4	1.92	0.66	8.52	0.0112[a]
b_{11}	−3.26	0.82	15.98	0.0013[a]
b_{22}	−1.92	0.85	5.11	0.0403[a]
b_{33}	−2.47	0.82	9.14	0.0091[a]
b_{44}	1.02	0.86	1.40	0.2560
b_1 b_2	0.0001	1.05	0.00	0.9999
b_1 b_3	2.55	1.05	5.97	0.0284[a]
b_1 b_4	0.58	1.05	0.30	0.5899
b_2 b_3	−2.25	1.05	4.63	0.0493[a]
b_2 b_4	0.29	1.31	0.048	0.8293
b_3 b_4	−1.15	1.05	1.22	0.2885

[a] p value less than 0.05 indicates model terms are significant.

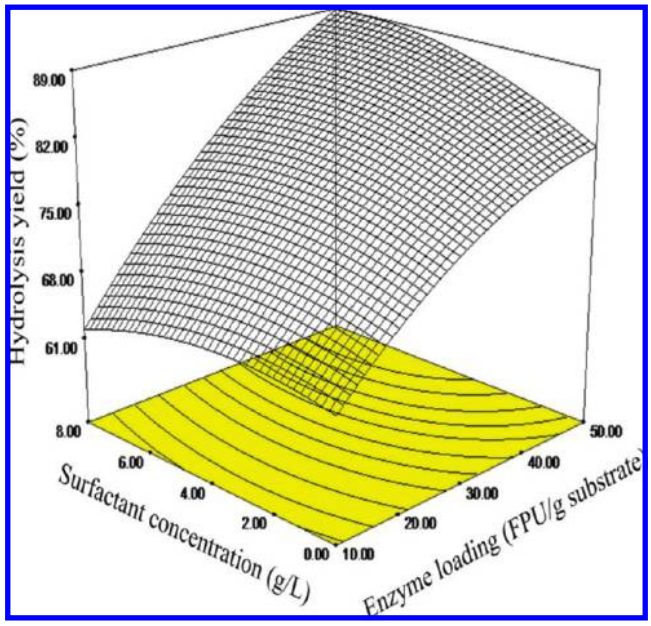

Figure 4. Response surface plot and contour plot of the combined effects of enzyme loading and surfactant concentration on the hydrolysis yield of WS with constant substrate concentration (50.00 g/L) and hydrolysis time (48 h).

effects on hydrolysis yield. The quadratic terms of X_1, X_2, and X_3 and interactions between X_1 and X_3, X_2 and X_3 also have significant effects on the hydrolysis yield. A statistically significant model only with significant terms can be written as follows:

$$Y = 79.60 + 10.93X_1 - 11.32X_2 + 1.92X_4 - 3.26X_1^2 - 1.92X_2^2 - 2.47X_3^2 + 2.55X_1X_3 - 2.25X_2X_3 \quad (3)$$

Effect of Variables on the Hydrolysis Yield. The response surface curves were plotted to examine the interaction of the variables and to determine the optimum level of each variable for maximum response.

The effects of enzyme loading and surfactant concentration on the hydrolysis yield of WS, when the other two factors were at their center points, are shown in Figure 4. When enzyme loading was at a low level, hydrolysis yield was low. Significant improvement in the hydrolysis yield could be obtained by increasing the amount of cellulase to some extent. When enzyme loading was at a low level, an increase in surfactant concentration only resulted in a slight increase in the hydrolysis yield. This may be due to the mechanism behind the surfactant addition to the enzymatic hydrolysis of lignocellulosic biomass: surfactant adsorbs the lignin and prevents unproductive binding of cellulase.[11] Therefore, with addition of surfactant, a high content of lignin often leads to a significant increase in conversion while a low lignin content results in a slight increase.[11] A relatively low lignin content (4.59%) in WS pretreated by alkaline peroxide could explain the slight effect of surfactant addition. When the enzyme loading increases to some extent, increasing surfactant concentration can result in a significant increase in hydrolysis yield, for example, at 50.00 FPU/g substrate of enzyme concentration, hydrolysis yield increases to 88.48% from 81.28% when surfactant concentration increases to 6.00 g/L from 0 g/L. A fixed substrate concentration requires a certain amount of cellulase to reach adsorption saturation for cellulose hydrolysis; further increase in the enzyme loading would result in more free cellulase in the reaction mixture. The presence of surfactant may have a stabilizing effect on these free cellulase to prevent its deactivity;[40,41] as a result, more active cellulase will be present for subsequent hydrolysis of WS; thus, hydrolysis yield could increase with addition of surfactant at a high enzyme concentration.

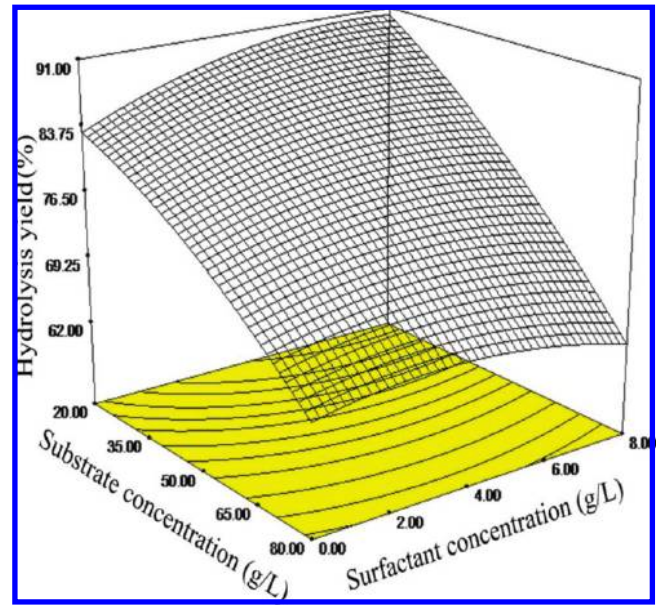

Figure 5. Response surface plot and contour plot of the combined effects of substrate concentration and surfactant concentration on the hydrolysis yield of WS with constant enzyme loading (30.00 FPU/g substrate) and hydrolysis time (48 h).

The effects of substrate loading and surfactant concentration on the hydrolysis yield of WS, when the other two factors were at their center points, are shown in Figure 5. Substrate concentration is considered to be one of the major factors affecting the conversion rate of enzymatic hydrolysis of cellulose. The hydrolysis yield decreases slowly with an increase in substrate concentration from 20.00 to 80.00 g/L. Previous work also showed that high substrate concentration resulted in low hydrolysis yield due to production inhibition, enzymatic inactivation, and a decrease in the reactivity of cellulosic substrate with proceeding of hydrolysis process.[42] The contour plot shows that a high conversion rate of WS was obtained at lower substrate concentration with addition of a high level of surfactant, while at a higher substrate concentration, intermediate levels of surfactant concentration are needed to obtain a high hydrolysis yield, and then any further increase in surfactant

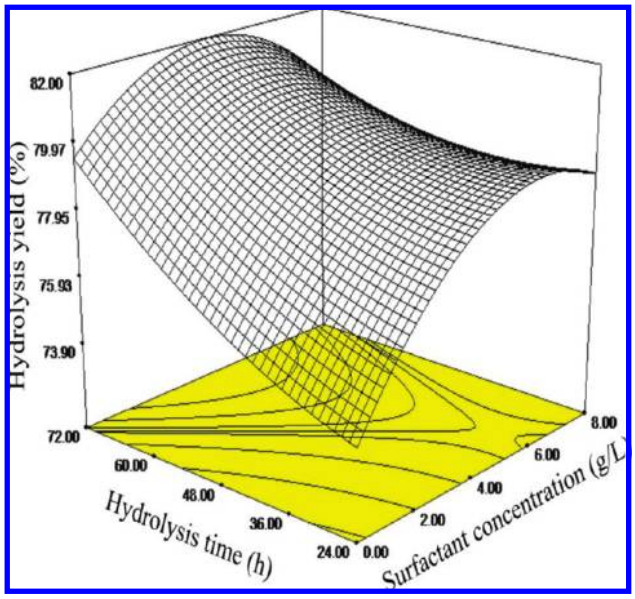

Figure 6. Response surface plot and contour plot of the combined effects of surfactant concentration and hydrolysis time on the hydrolysis yield of WS with constant enzyme loading (30.00 FPU/g substrate) and substrate concentration (50.00 g/L).

concentration would lead to a slight decrease in hydrolysis yield. This may be caused by the decreased enzymatic activity due to the formation of reverse micelles at high Tween 80 concentration (much higher than its critical micelle concentration, CMC).[43]

The effects of surfactant concentration and hydrolysis time on the hydrolysis yield of WS, when the other two factors were at their center points, are shown in Figure 6. As can be seen from Figure 6, a pronounced effect of hydrolysis time on enzymatic digestion of pretreated WS was observed at a low level of surfactant concentration, while at a high concentration of surfactant, prolonging reaction time within the tested range only resulted in a small increase in hydrolysis of polysaccharides contained in pretreated WS. For example, conversion of carbohydrate polymer increased from 75.39% to 80.04% at 1.00 g/L of surfactant concentration, but only increased from 79.23% to 80.75% at 7.00 g/L of surfactant concentration when increasing reaction time from 24 to 72 h. This phenomenon could be explained by the effect of nonionic surfactant on enzymatic hydrolysis of pretreated lignocellulosic materials. When the surfactant concentration was set at a low level, the slight enhancement of enzymatic hydrolysis of pretreated WS by surfactant addition caused a low degree of carbohydrate conversion and produced a small amount of end product at the initial hydrolysis stage (<24 h); therefore, a considerable increase in the hydrolysis yield was observed in the following period of 24−72 h. When increasing surfactant concentration to a high level, the beneficial effect of adding surfactant to the reaction mixture on enzymatic hydrolysis would result in a rapid hydrolysis of pretreated substrate at a short reaction time (<24 h); then the related product inhibition resulting from buildup of hydrolysis products would be expected, thus leading to a slight increase in the hydrolysis yield with further increasing reaction time from 24 to 72 h.

Confirmation Experiments and Adequacy of the Models. The second-order polynomial regression equation obtained from the experimental data can be used to predict the hydrolysis rate at any enzyme loading, substrate concentration, surfactant concentration, and hydrolysis time within the range of the experimental design. In order to confirm the validity of the

Table 6. Confirmation Experiments

				hydrolysis rate (%)			
X_1 (FPU/g substrate)	X_2 (g/L)	X_3 (g/L)	X_4 (h)	actual	predicted	residual	error (%)
30.00	50.00	4.00	48	76.80	79.60	−2.80	3.65
10.00	50.00	0	48	67.20	64.20	3.00	4.46
50.00	50.00	4.00	72	87.61	90.80	−3.19	3.64
40.00	22.00	6.76	72	97.01	99.14	−2.13	2.20

statistical experimental strategies and to gain a better understanding of the hydrolysis yield of WS, four confirmation runs were performed. The conditions are listed in Table 6. The hydrolysis conditions for the first three confirmation experiments were among the conditions that were used in Table 2, while the fourth experiment was performed under the hydrolysis condition that has not been conducted but was within the range of the levels defined previously. The result shows that under the following conditions: cellulase loading 40.00 FPU/g, substrate concentration 22.00 g/L, surfactant concentration 6.76 g/L, and hydrolysis time 72 h, the hydrolysis yield nearly reached the theoretical limit. Xu et al. performed enzymatic hydrolysis of ammonia liquor pretreated soybean straw using an enzyme loading of 50 FPU/g substrate and obtained a maximum hydrolysis yield of 51.22% at 5% (w/v) substrate concentration for 36 h.[44] Sun and Chen found that an enzyme loading of 44 FPU/g substrate was necessary to have a hydrolysis yield of 92% from enzymatic digestion of wheat straw pretreated by atmospheric glycerol autocatalysis at 2% (w/w) substrate concentration after 48 h.[23] Mussatto et al. concluded that 45 FPU/g substrate was an enzyme loading enough to achieve complete conversion of cellulose from brewer's spent grain pretreated by a two-step chemical pretreatment process using dilute acid and alkali at 2% (w/v) substrate concentration for 96 h.[45] In the literature[23,44,45] and also in the present work, *T. reesei* cellulase preparation without supplementation of additional β-glucosidase was used for enzymatic hydrolysis, due to the small amount of β-glucosidase activity contained in cellulase preparation; high cellulase dosage with a reasonable amount of β-glucosidase activity was required to prevent the buildup of end-product cellobiose and to achieve high hydrolysis yield.

The predicted hydrolysis rates of the selected experiments could be obtained through the point prediction capability of the software. The predicted values and actual experimental values were compared, and the residual and percentage error were calculated. All values are listed in Table 6. The percentage error between the actual and predicted value for hydrolysis rates are observed to vary from 2.20% to 4.46%. Therefore, the empirical models developed were reasonably accurate, and the RSM analysis is indeed a useful technique to predict and optimize the hydrolysis of lignocellulosic feedstocks. Usually, it is important to check the adequacy of the model to ensure that it provides maximum approximation on the relationship between factors and response. The residuals from the least squares are an important tool for judging the model adequacy.[46] Normal probability was checked by plotting the normal probability plot of residuals. The normality assumption is satisfactory as normal residuals fall along a straight line as shown in Figure 7. Figure 8 is the plot of residuals versus the predicted response. The residual plots of the model are randomly distributed without any trends. This result indicates good predictions of maximum response along with constant variance and adequacy of the quadratic models.[47]

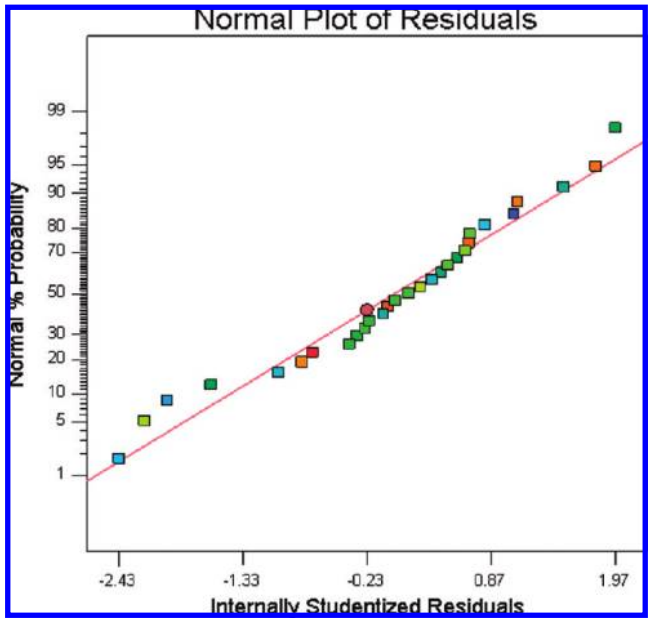

Figure 7. Normal probability of internally studentized residuals for WS hydrolysis.

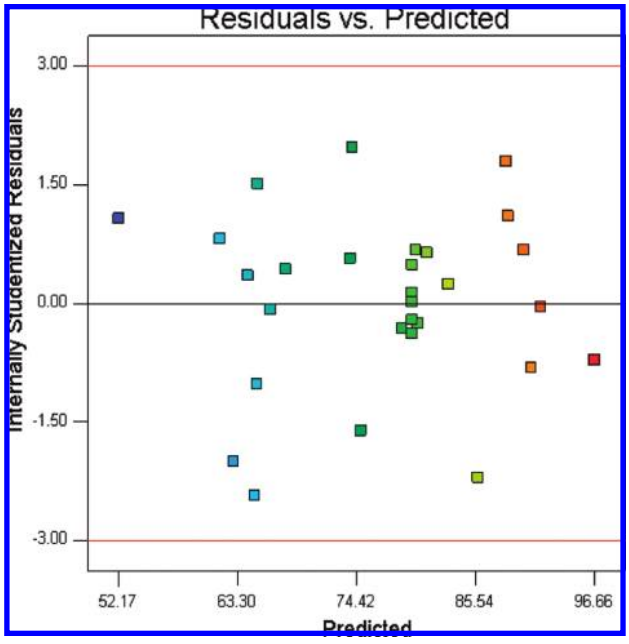

Figure 8. Plot of internally studentized residuals vs predicted response.

4. Conclusions

The pretreatment of WS by alkaline peroxide produced substrate consisting of 60.17% cellulose, 29.53% hemicelluloses, and 4.59% lignin. The decrease in the content of lignin and hemicellulose could facilitate the process of enzymatic hydrolysis. The RSM was performed to investigate the enzymatic hydrolysis of pretreated WS for production of reducing sugars. The experimental results showed that both enzyme loading and substrate concentration had interactions with surfactant concentration, and the interactions had significant effects on the hydrolysis of polysaccharides contained in the pretreated WS. According to the regression equation obtained by RSM, nearly the theoretical hydrolysis yield was obtained under the following conditions: cellulase loading 40.00 FPU/g, substrate concentration 22.00 g/L, surfactant concentration 6.76 g/L, hydrolysis time 72 h. Therefore, RSM with the Box−Behnken design is useful for identifying the important factors influencing enzymatic conversion of pretreated WS carbohydrate and predicting the enzymatic hydrolysis process of lignocellulosic substrates.

Acknowledgment

The authors would like to thank the Chinese Academy of Sciences' Knowledge Innovation Program for financial support (grant no. KSCX1-YW-11D2).

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Received for review November 5, 2008
Revised manuscript received June 5, 2009
Accepted June 10, 2009

IE8016863