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Statistical Optimization of Recycled-Paper Enzymatic Hydrolysis for Simultaneous Saccharification and Fermentation Via Central Composite Design

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Abstract A central composite design of the response surface methodology (RSM) was employed to study the effects of temperature, enzyme concentration, and stirring rate on recycled-paper enzymatic hydrolysis. Among the three variables, temperature and enzyme concentration significantly affected the conversion efficiency of substrate, whereas stirring rate was not effective. A quadratic polynomial equation was obtained for enzymatic hydrolysis by multiple regression analysis using RSM. The results of validation experiments were coincident with the predicted model. The optimum conditions for enzymatic hydrolysis were temperature, enzyme concentration, and stirring rate of 43.1 °C, 20 FPU g^{-1} substrate, and 145 rpm, respectively. In the subsequent simultaneous saccharification and fermentation (SSF) experiment under the optimum conditions, the highest 28.7 g ethanol l^{-1} was reached in the fed-batch SSF when 5% (*w*/*v*) substrate concentration was used initially, and another 5% added after 12 h fermentation. This ethanol output corresponded to 77.7% of the theoretical yield based on the glucose content in the raw material.

Keywords Enzymatic hydrolysis · Cellulose · Response surface methodology (RSM) · Central composite design (CCD)

Introduction

Due to energy crisis and environmental problems, the recovery and utilization of waste papers is increasing all over the world. Recycled paper offers an opportunity as raw materials for bio-products for its high lignocellulosic content [1].

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This attractive lignocellulosic substrate requires hydrolyzing all of its major components (cellulose and hemicellulose) to glucose and other simple fermentable sugars and further converting them to fuels and chemicals, which has been considered to be an attractive route for ethanol production [2, 3]. Compared with acid hydrolysis, enzymatic hydrolysis is milder and more specific [4, 5]. Enzymatic hydrolysis is affected by many factors such as temperature, enzyme concentration, etc. These factors often interact with one another [6, 7]. The conventional method (changing one independent variable while maintaining all of the others at a fixed level) to obtain the optimization of enzymatic hydrolysis is extremely time consuming. Response surface methodology (RSM) is a time- and labor-saving method, which reveals the interaction between the factors and seeks the optimum levels [8, 9]. RSM mainly includes central composite design, Box–Behnken design, one-factor design, D-optimal design, user-defined design, and historical data design. In this study, the central composite design (CCD) was adopted to optimize the levels of temperature, enzyme concentration, and stirring rate in the enzymatic hydrolysis process. The results could be used successfully in the prehydrolysis process for simultaneous saccharification and fermentation (SSF).

Materials and Methods

Materials

Recycled paper, kindly provided by CTA-TEX Chemical Co. Ltd. in China, was used as the substrate in the RSM experiments and SSF assays. The compositions of recycled paper were determined in our laboratory according to corresponding Chinese National standards. The data are shown in Table 1.

Enzymatic Hydrolysis

Enzymatic hydrolysis experiments were carried out in 50-ml stoppered conical flasks with 20 ml medium in an air-bath shaker. The cellulase used in the experiments was Cellulase ZC-1700, which was produced by CTA-TEX Chemical Co. Ltd. in China. The cellulase activity was determined by the method recommended by Ghose [11] and expressed in terms of filter paper units (FPU). One FPU was defined as the amount of enzyme capable of producing 1 μmol of reducing sugars in 1 min. Citrate buffer was used to adjust the pH to 4.5. The substrate concentration was in the presence of 5% (*w*/*v*) medium.

Table 1 Compositions of recycled paper.

Component (%, *w*/*w*)	Values (%)	Methods
Cellulose	73.35±0.17	Nitric acid–ethanol method
Holocellulose	94.00±0.26	GB/T 2677.10-1995
Hemicellulose	20.65±0.09	Calculated value
Klason lignin	1.00±0.02	GB/T 2677.8-1994
Acid-soluble lignin	0.16±0.02	GB/T 747-2003
Total lignin	1.16±0.04	GB/T 2677.8-1994, GB/T 10337-1989

Cellulose content was determined by the nitric acid–ethanol method [10]. Hemicellulose is calculated by difference between hollocellulose and cellulose. The values were the mean of four independent samples

Microorganism and Culture Conditions for SSF

Kluyveromyces marxianus DW08 was grown on the preculture medium containing 2 g $(NH_4)_2SO_4$ l^{-1}, 5 g KH_2PO_4 l^{-1}, 1 g $MgSO_4$ l^{-1}, 0.2 g $CaCl_2$ l^{-1}, 5 g yeast extract l^{-1}, and 30 g glucose l^{-1}. The recycled paper, at a solid loading of 5–10% (*w*/*v*), supplemented with 2 g $(NH_4)_2SO_4$ l^{-1}, 5 g KH_2PO_4 l^{-1}, 1 g $MgSO_4$ l^{-1}, 0.2 g $CaCl_2$ l^{-1}, and 5 g yeast extract l^{-1} was used for fermentation medium. The medium was adjusted to pH 4.5 with citrate buffer. The cellulase solutions were not sterilized and added during the prehydrolysis process of SSF experiments.

The seed cells were prepared in a 250-ml flask containing 50 ml of preculture medium at 35 °C in an air-bath shaker at 150 rpm for 12 h. Also, 250-ml flasks containing 50 ml fermentation medium were used in SSF with a 5% (*v*/*v*) inoculum.

Analytical Methods

After the enzymatic hydrolysis experiments, the residues were centrifuged at 10,000 rpm for 10 min. The solid fractions were washed with water to remove inorganic compounds and centrifuged again and then were dried at 80 °C to a constant weight. The conversion efficiency of substrate (CES) was calculated according to:

$$\mathrm{CES} = \mathrm{substrate_R(g)}/\mathrm{substrate_I(g)} \times 100\%$$

where $\mathrm{substrate_I}$ is the initial weight of the substrate and $\mathrm{substrate_R}$ is the weight of the residue after enzymatic hydrolysis.

The liquid samples after SSF were analyzed by HPLC, equipped with RI detectors. The concentrations of glucose, xylose, galactose, mannose, and arabinose were determined using refractive index detector and Aminex HPX-87P column at 85 °C with H_2O as mobile phase at 1 ml min^{-1}. Ethanol was analyzed using refractive index detector and Aminex HPX-87H column at 65 °C with 5 mM H_2SO_4 as mobile phase at 0.8 ml min^{-1}.

Experimental Design

CCD-Uniform Design with a rotatable type of axial scale was used to investigate the significance of the effects of temperature, enzyme concentration and stirring rate. The experiments were designed by using the Design-Expert 7.0.3 Trial (State Ease Inc., Minneapolis, MN, USA). The lowest and the highest levels of variables are given in Table 2.

A three-level, three-factor factorial central composite design and six replicates at the center points leading to 20 runs was employed for the optimization of the enzymatic hydrolysis. The variables were coded according to the following equation:

$$x_i = (X_i - \mathrm{X_0})/\Delta X_i \quad i = 1, 2, \ldots, k \tag{1}$$

Table 2 Variables and experimental design levels for response surface.

Variables	Symbol	Range and levels		
		−1	0	1
Temperature (°C)	X_1	35	40	45
Enzyme concentration (FPU)	X_2	10	15	20
Stirring rate (rpm)	X_3	120	140	160

where x_i and X_i are the dimensionless and the actual values of the independent variable i, X_0 is the actual value of the independent variable at the center point, and ΔX_i is the step change of X_i corresponding to a unit variation of the dimensionless value. The quadratic model for predicting the optimal point was expressed as Eq. (2):

$$y_i = b_0 + \sum b_i x_i + \sum b_{ii} x_i^2 + \sum b_{ij} x_i x_j \quad i = 1, 2, \ldots, k; j = 1, 2, \ldots, k; i \neq j \qquad (2)$$

where y_i is the predicted response, b_0 is the interception coefficient, b_i is the linear term, b_{ii} is the quadratic term, and b_{ij} is the interaction term.

Results

Optimization of the Reaction Conditions

Effects of the three variables, including temperature, enzyme concentration, and stirring rate, on CES were studied. The experimental design matrix is presented in Tables 2 and 3. All experiments were carried out in triplicate and data were expressed as average values. Each enzymatic hydrolysis process was stopped at 48 h. CES was selected as the response to different cycles of the runs.

The results of the second-order response surface models for the CES in the form of analysis of variance (ANOVA) are given in Tables 4 and 5, respectively. Using the designed

Table 3 Experimental design with real value and predicted value of CES.

Runs	X_1	X_2	X_3	CES (%)	
				Experimental	Predicted
1	−1	−1	−1	0.303±0.011	0.303
2	−1	−1	1	0.309±0.011	0.312
3	−1	1	−1	0.409±0.025	0.401
4	−1	1	1	0.403±0.036	0.407
5	−1.68	0	0	0.304±0.045	0.309
6	0	−1.68	0	0.334±0.026	0.325
7	0	0	0	0.373±0.043	0.437
8	0	0	0	0.454±0.038	0.437
9	0	0	0	0.440±0.024	0.437
10	0	0	0	0.431±0.015	0.437
11	0	0	0	0.457±0.041	0.437
12	0	0	0	0.464±0.043	0.437
13	0	0	−1.68	0.404±0.040	0.407
14	0	0	1.68	0.436±0.015	0.422
15	0	1.68	0	0.473±0.043	0.471
16	1	−1	−1	0.377±0.012	0.381
17	1	−1	1	0.377±0.016	0.393
18	1	1	−1	0.455±0.010	0.460
19	1	1	1	0.460±0.031	0.468
20	1.68	0	0	0.442±0.010	0.426

Table 4 ANOVA for response surface quadratic model.

Source	Sum of squares	*df*	Mean square	*F* value	Probe >*F*
Model	0.054	9	5.985E−003	9.10	0.0009
Residual	6.576E−003	10	6.576E−004	–	–
Lack of fit	1.019E−003	5	2.038E−004	0.18	0.9569
Pure error	5.558E−003	5	1.112E−003	–	–
Cor total	0.60	19	–	–	–

CV=6.33%; R^2 =0.8912; Pred R^2 =0.7389; Adj R^2 =0.7933; Adeq precision=9.291

experimental data (Table 3), the second-order polynomial model for the yield of CES in terms of coded factors is shown as the following equation:

$$Y = 0.44 + 0.035X_1 + 0.044X_2 + 4.307\text{E} - 003X_3 - 4.875\text{E} - 003\,X_1X_2 + 6.250\text{E} - 004X_1X_3 - 8.750\text{E} - 004\,X_1X_3 - 0.025X_1^2 - 0.014X_2^2 - 7.928\text{E} - 003X_3^2 \quad (3)$$

where Y is the predicted CES, X_1 is temperature, X_2 is enzyme concentration, and X_3 is stirring rate.

As shown in Table 4, the "Model *F* value" of 9.10 implies that the model is significant. The fitness of the model is examined by determination coefficient (R^2=0.8912), which implies that the sample variation of more than 89% is attributed to the variables and only 10.88% of the total variance could not be explained by the model. The predicted determination coefficient (Pred R^2=0.7389) is in reasonable agreement with the adjusted determination coefficient (Adj R^2=0.7933), which is also satisfactory to confirm the fitness of the model. A lower value of coefficient of variation (CV=6.33%) shows the experiments conducted are precise and reliable. "Adeq Precision" measures the signal-to-noise ratio. A ratio greater than 4 is desirable. The ratio of 9.291 indicates an adequate signal, which implies this model could be used to navigate the design space [12–16].

Table 5 shows the significance of each coefficient, which is measured by *t* test and *P* value. The larger the magnitude of *t* test and smaller the *P* values are, the more significant the corresponding coefficient is [17]. Values of "Prob > *F*" less than 0.0500 indicate model terms are significant whereas values greater than 0.1000 indicate the model terms are not significant. In this case, temperature and enzyme concentration had significant effects on

Table 5 Results of regression analysis of a second-order polynomial model.

Term	Coefficients estimated	Standard error	*t* statistic	*P* value
Intercept	0.44	0.010	44	0.0009
X_1	0.035	6.939E−003	5.043954	0.0005
X_2	0.044	6.939E−003	6.340971	<0.0001
X_3	4.307E−003	6.939E−003	0.620695	0.5487
$X_1\ X_2$	−4.875E−003	9.067E−003	−0.53766	0.6026
$X_1\ X_3$	6.250E−004	9.067E−003	0.068931	0.9464
$X_2\ X_3$	−8.750E−004	9.067E−003	−0.0965	0.9250
X_1^2	−0.025	6.755E−003	−3.70096	0.0046
X_2^2	−0.014	6.755E−003	−2.07254	0.0690
X_3^2	−7.928E−003	6.755E−003	−1.17365	0.2678

CES whereas the effect of the string rate was not significant. There are no significant interactions between any two factors. The quadric effect of temperature is significant while the quadric effect of stirring rate is not significant.

The 2D contour plot and 3D response surface are generally the graphical representation of the regression equation. Figures 1, 2 and 3 represent the 2D contour plots for the optimal conditions of enzymatic hydrolysis. Each figure presents the effect of two variables on CES. From the analysis of the contour plots, which was conducted by the Design-Expert software automatically, the optimum combinations were temperature, enzyme concentration, and stirring rate of 43.1 °C, 20 FPU, and 145 rpm. Under the optimum conditions, the maximum conversion efficiency predicted by the model was 0.476 g g^{-1} substrate. The validation experiments under the optimum conditions obtained the conversion efficiency of 0.472±0.016 g g^{-1} substrate, which was coincident with the prediction.

SSF

The optimum conditions obtained were further applied in the prehydrolysis process of SSF experiments as the reference described [18–21]. In the first step of SSF, the recycled paper was prehydrolyzed under the optimum conditions for 12 h. After prehydrolysis, *K. marxianus*, a thermotolerant yeast and capable of growth and producing ethanol with a high yield at 40–45 °C, was added to the fermentation medium. The SSF experiments were performed under non-aseptic conditions with uncontrolled pH starting at 4.5 at the beginning of the fermentation and dropping steadily to 4.2 at the end. The highest 28.7 g ethanol l^{-1} was reached in the fed-batch SSF when 5% (*w*/*v*) substrate concentration was used initially, and another 5% added after 12 h fermentation (Table 6). This ethanol output corresponded to 77.7% of theoretical ethanol yield based on the glucose content in the raw material.

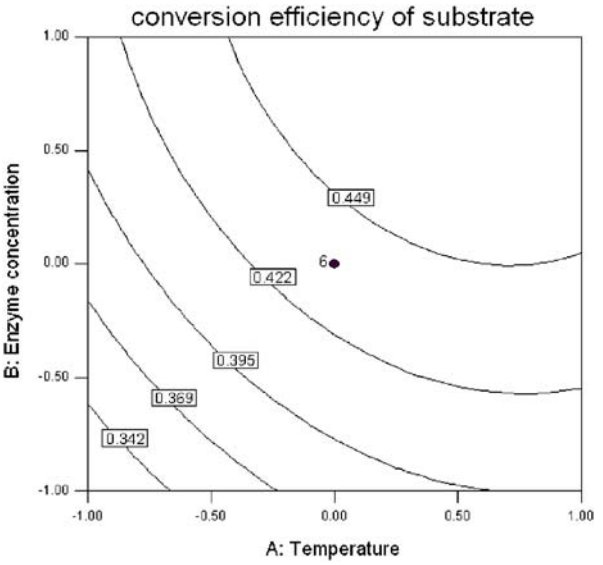

Fig. 1 Contour plot of the combined effects of temperature and enzyme concentration: Design point (●), the number *6* in the figure corresponds to six replications in center point

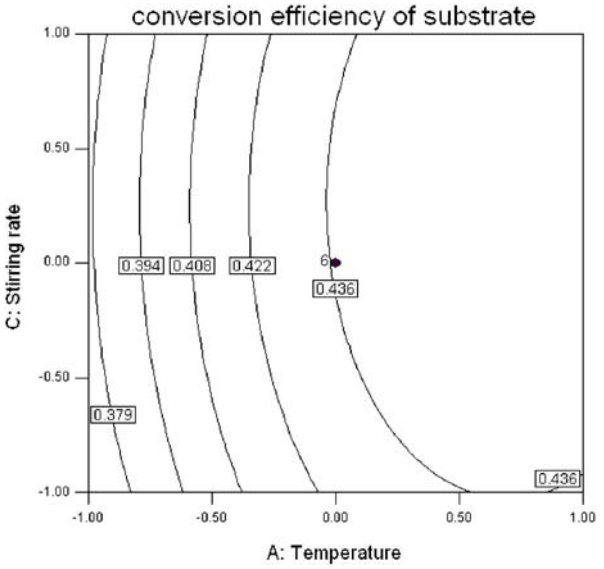

Fig. 2 Contour plot of the combined effects of temperature and stirring rate. Design point (●), the number *6* in the figure corresponds to six replications in center point

Discussion

Operation parameter optimization by the traditional one-factor-at-a-time technique requires a considerable amount of work and time. An alternate strategy is a statistical approach, such as RSM, involving a minimum number of experiments for a large number of factors. RSM has been shown to optimize the process in many works, such as the 1, 3-propanediol culture conditions [17], production of thermal stable β-glucanase [9] and enzymatic hydrolysis of maize starch [12], etc. This method has also been used to optimize the enzymatic hydrolysis

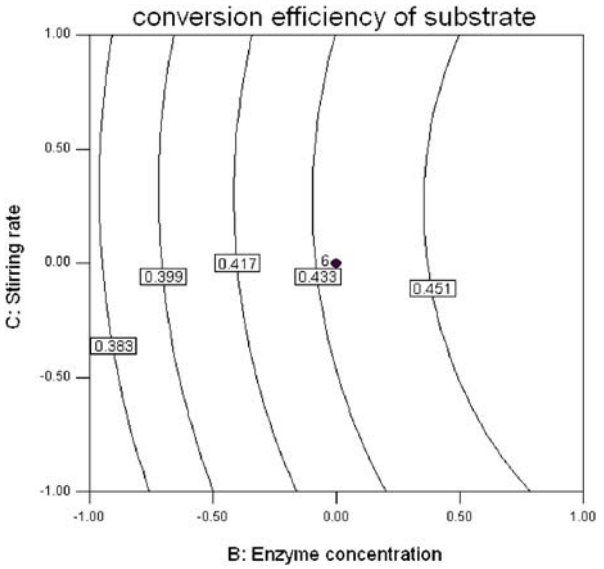

Fig. 3 Contour plot of the combined effects of enzyme concentration and stirring rate. Design point (●), the number *6* in the figure corresponds to six replications in center point

Table 6 Ethanol concentration and SSF yield (expressed as percentage of theoretical yield) with different substrate loading.

Substrate loading (%, *w*/*v*)	Glucose before SSF (g l^{-1})	Glucose after SSF (g l^{-1})	Xylose (g l^{-1})	Arabinose (g l^{-1})	Galactose (g l^{-1})	Ethanol (g l^{-1})	Percentage of theoretical yield (%)
5	15.5	0.1	4.1	3.1	0.5	13.4	71.7±0.3
7.5	14.2	0	7.2	3.2	0.5	19.5	73.7±0.7
10	10.4	0	9	4.8	0.7	16.7	57.5±1.1
5+5 (12 h)	15.3	0	9.4	5.7	0.8	28.7	77.7±0.4

process earlier in the work by Lu et al. [7], who studied the effect of enzyme concentration, stirring rate, temperature, pH, and time on enzymatic hydrolysis of corn stover. In this study, the effects of temperature, enzyme concentration, and stirring rate on recycled-paper enzymatic hydrolysis were studied using RSM. The optimum conditions could be used successfully in the prehydrolysis process for simultaneous saccharification and fermentation.

The predicted maximum conversion efficiency was 0.476 g g^{-1} substrate, and there was still 0.464 g holocellulose that remained unhydrolyzed. Compared with a similar study by Ballesteros et al. [22], who obtained the highest 17.7 ethanol l^{-1} and 79.7% ethanol yield with enzyme loading of 45 FPU, higher ethanol concentration was obtained in this study and the enzyme concentration we selected was 20 FPU g^{-1} substrate. Theoretically, the higher the enzyme concentration is, the higher the conversion efficiency of the substrate is. However, high enzyme loading is expensive and uneconomical. Moreover, the recycled paper used in this experiment was not pretreated, which might cause insufficient hydrolysis efficiency. The enzymatic digestibility may be increased if the recycled paper was pretreated to sludge.

Compared with the substrate loading of 5%, 7.5%, and 10%, the substance added in two portions could overcome the effects of the saturation limit on the activity of the enzymes. The highest 28.7 g ethanol l^{-1} was reached in the fed-batch SSF when 5% (*w*/*v*) substrate concentration was used initially, and another 5% added after 12 h fermentation. Furthermore, if the xylose, arabinose, and galactose presented in the broth at the end of the SSF could be fermented to ethanol, another 8.0 g ethanol l^{-1} could theoretically be produced (0.51 g ethanol/g pentose). Similar trends were observed by Varga and Klinke [18], who studied the effect of the substrate concentration on the ethanol yield. When the dry matter content was increased to 12% dry matter, no fermentation products could be seen at all. However, in the way that the DM was added to the reaction partly during enzymatic prehydrolysis, the DM content could be increased efficiently to 17% with a high ethanol yield.

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