RESPONSE SURFACE OPTIMIZATION OF ENZYMATIC HYDROLYSIS PROCESS OF WET OXIDATION PRETREATED WOOD PULP WASTE

SHANSHAN LIU[*,**] and QIANG WANG[*,**]

[*]*Key Laboratory of Paper Science and Technology of Ministry of Education, Qilu University of Technology, Jinan, Shandong Province, 250353, China*
[**]*Jiangsu Provincial Key Laboratory of Pulp and Paper Science and Technology, Nanjing Forestry University, Nanjing, Jiangsu Province, 210037, China*
✉ *Corresponding author: Q. Wang, wangqiang8303@163.com*

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The bioconversion of lignocellulosic waste (known as wood pulp waste) from the pulping production process into fermentable sugar is a promising way to implement the integrated forest biorefinery concept. The enzymatic hydrolysis process is a feasible step for the bioconversion process. In this paper, the enzymatic hydrolysis process was optimized by a two-level three factor (2^3) full factorial central composite design through varying temperature, time and enzyme loading. The results showed that the optimum conditions were: temperature of 50 °C, time of 48 h and enzyme loading of 35 FPU/g substrate, which gave an 85.4% of cellulose conversion ratio. Additionally, the X-ray diffraction (X-RD) and scanning electronic microscopy (SEM)of the substrates showed decreases of the crystallinity index and degradation of cellulose after enzymatic hydrolysis. The optimization of enzymatic hydrolysis provides an economical and efficient approach to implement the bioconversion process of pretreated wood pulp waste.

Keywords: bioconversion, wood pulp waste, enzymatic hydrolysis, cellulose conversion ratio

INTRODUCTION

Bioconversion of lignocellulosic waste into a value-added product, *e.g.* fermentable sugar, is a promising approach to face the depleting non-renewable resources.[1,2] Wood pulp waste, a kind of lignocellulosic waste,is generated from the washing stage of P-RC APMP (pre-conditioning refiner chemical alkaline peroxide mechanical pulping) production process of the pulping industry, which is currently disposed into landfill at the cost of capital and land.[3] The utilization of these lignocellulosic wastes for the bioconversion process fits well into the concept of integrated forest biorefinery.

The bioconversion process includes three key steps: pretreatment, hydrolysis and fermentation.[4-6] The compact physical and chemical structure of lignocellulose (namely, its recalcitrance) prevents the access of the hydrolysis agent to cellulose, therefore, a pretreatment is a prerequisite to overcome the recalcitrance of lignocellulose.[7] The wet oxidation (WO) pretreatment was employed to pretreat the wood pulp waste in the present paper, using water and oxygen under alkaline conditions. The advantage of the WO pretreatment is the low concentration of toxic furaldehydes and phenol aldehydes, which has been confirmed by previous studies.[8,9]

Apart from the pretreatment, the hydrolysis has been identified as one of the most costly steps in the bioconversion process.[10] The hydrolysis of the substrate can be divided into two categories, *i.e.* acid hydrolysis and enzyme hydrolysis.[11] The drawback of the former hydrolysis consists in the release of toxic chemicals, which hinder the subsequent fermentation step.[12,13] Fortunately, the latter hydrolysis is a highly efficient, energy-saving approach, and benefits the following fermentation, hence it has been widely adopted. The enzymatic hydrolysis process involves several parameters, *i.e.* temperature, hydrolysis time and enzyme loading.

Although many papers have been published on the enzymatic hydrolysis of a pretreated substrate, the optimum conditions are different for specific enzymes. Moreover, the optimization of enzymatic

hydrolysis was an effective way to reduce the hydrolysis cost. For this purpose, the statistical technique of response surface methodology (RSM) was employed here to analyze the effects of several independent variables.[14-16] The parameters of temperature, time and enzyme loading were varied to obtain the highest cellulose conversion ratio. The X-RD and SEM were carried out to characterize the enzymatic hydrolysis residuals.

EXPERIMENTAL

Materials and chemicals

The wood pulp waste was collected from the washing stage of P-RC APMP production process, Shandong Chenming Paper Co., Ltd., China.

Cellulase L-10 used in this work was provided by KDN BIOTECH GROUP, Tsingtao, China. The cellulase activity was150 FPU/mL and was measured by filter paper enzyme activity according to a literature report.[17]All chemicals were from commercial sources.

Pretreatment

The WO pretreatment was carried out in an oxygen delignification reactor (inside volume of 3 L), which was heated via an air bath. The pretreatment conditions were: solid/liquid ratio of 1/15, initial pH 10, oxygen pressure of 12 bar, temperature of 195 °C, time of 15 min. Once the pretreatment was completed, the substrate was separated by filter paper, and then stored in a polythene bag after air drying.

Enzymatic hydrolysis

Enzymatic hydrolysis of the substrates (0.5 g dry basis) was carried out in an Erlenmeyer flask in ashaking incubator (160rpm).The substrate concentration was 2% in 50 mL of acetate buffer (pH 4.8). The parameters were varied in the following ranges: temperature between 45-55 °C, time – 24-72 h, enzyme loading– 25-45 FPU/g substrate. At the end of the hydrolysis, the residual was separated from the hydrolysate by filtration, and placed in a fridge until further analysis.

Response surface methodology (RSM)

The optimization approach was based on 2^3 full factorial central composite designs (CCD) for RSM and conducted using Design Expert (Version 7.1). A set of three independent variables, *i.e.* temperature (X_1,°C), time (X_2, h) and enzyme loading (X_3, FPU/g)were identified to investigate the influence on the cellulose conversion. The experimental range of the selected process variables with their units and notation is shown in Table 1.

The response variable, *Yc* (cellulose conversion ratio) can be expressed as a function of the independent process variables according to the following response surface quadratic model (Eq.1):

$$Y_C = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1} \sum_{j=1+1} \beta_{ij} x_i x_j + \xi \quad (1)$$

whereβ_0 is the constant coefficient, β_i, β_{ii} and β_{ij} are the coefficients for the linear, quadratic and interaction effect, x_i and x_j are the independent variables and ξ is the error.

Analytical methods

The chemical composition of the wood pulp waste and pretreated substrate was analyzed according to TAPPI methods.

The reducing sugar content in the hydrolyzate was detected by the DNS method at a wavelength of 540 nm by a UV-vis spectrophotometer (T6).[18,19] The cellulose conversion ratio (*Yc*, %) was calculated based on Eq. 2:

$$Y_C = \frac{m_{RS}/1.11}{m_C} \times 100\% = \frac{CV/1.11}{m_0 \omega} \times n \times 100\% \quad (2)$$

where m_{RS} is the weight of reducing sugar (g), 1.11 is the molar mass ratio of glucose ($C_6H_{12}O_6$) and cellulose ($(C_6H_{10}O_5)_n$), m_c is the weight of cellulose in pretreated substrate (g), *C* is the concentration of reducing sugar (g L^{-1}), *V* is the total volume of the hydrolysate (L), *n* is the number of dilution times, m_0 is the substrate weight (g), and ω is the cellulose content (%).

Table 1
Experimental range and levels of independent process variables

Factor	Unit	Code	Level		
			-1	0	1
Temperature	°C	X_1	45	50	55
Time	h	X_2	24	48	72
Enzyme loading	FPU/g	X_3	25	35	45

The components of the hydrolyzate were detected by a HPLCsystem (Agilent 1100) equipped with an Aminex HPX-87H column. The column temperature was set at 63 °C. 10μL of sample was eluted at a rate of 0.6 ml/min with sulfuric acid (4 mmol/L). The sample was hydrolyzed under the following conditions: 4% (w/w)H_2SO_4, temperature of 121 °C for 1 h.

The surface morphology of the solid residue was observed by SEM (S-3400, Toshiba Corporation, Japan) with an accelerating voltage of 10 KeV. Prior to the test, the samples were sputter-coated with platinum for good conductivity.

The crystallinity was measured by X-RD (D8-FOCUS, Bruker Optics, Germany) with Cu Kα radiation (λ=0.154 nm) at 10 kV. X-RD data were collected at 2θ=10-60° at a scan rate of 2°/min. The sample was ground to 80-120 mesh size. The crystallinity (X_c) was calculated based on Eq.3:

$$X_c = \frac{A_{cr}}{A_{cr} + A_{am}} \times 100\% \quad (3)$$

where A_{cr} and A_{am} are the integrated area of the crystalline and amorphous phases, respectively.

RESULTS AND DISCUSSION

Material and pretreated substrate

The chemical composition of the wood pulp waste and WO pretreated substrate is listed in Table 2. As can be seen, the raw material was rich in carbohydrates, which can be used as a feedstock candidate of the bioconversion process. After the WO pretreatment, the composition of the substrate was changed pronouncedly. The hemicellulose was substantially removed (92.5%, based on pentosan), Klason lignin was removed by 42.4%, and part of the cellulose (26.2%) was degraded and dissolved into the hydrolysate. These composition changes can affect the compact cell wall and open additional channels, hence facilitating the subsequent enzymatic hydrolysis process. Varga *et al.*[9] reported that a pretreatment substrate yield of 48.6% was obtained with a cellulose content of 69.7%, lignin content of 19.2% and hemicellulose content of 7.1%, when using alkaline wet oxidation to pretreat corn stover for biofuel production.

Response surface quadratic model

A total of 15 enzymatic hydrolysis experiments were performed under the conditions listed in Table 3. It can be seen that the cellulose conversion ratio was in the range of 73.3-86.7% under varied conditions, suggesting that the enzymatic hydrolysis conditions had an obvious effect on the cellulose conversion ratio. Zhu *et al.*[20] reported that they achieved a cellulose conversion ratio in the range of 68-92% for enzymatic hydrolysis of sodium bisulfite pretreated spruce. Huo *et al.*[18] employed alkali impregnation and refining pretreatment to enhance enzymatic hydrolysis of eucalyptus and achieved a reducing sugar yield in the range of 65-90%.

Table 2
Chemical composition of wood pulp waste and WO pretreated substrate

	Cellulose (g)	Klason lignin (g)	Pentosan (g)	Ash (g)	Benzene-ethanol extractives (g)	Sum (g)	Total (g)
Wood pulp waste	44.3	23.6	18.7	2.6	1.1	90.3	100.0
Substrate	32.7	13.5	1.4	0.1	0.4	48.1	51.7

Table 3
Cellulose conversion ratio by the central composite design

Sample	Temperature (°C)	Time (h)	Enzyme loading (FPU/g)	Cellulose conversion ratio (%)
1	45	24	25	74.4
2	55	24	25	73.3
3	45	72	25	80.1
4	55	72	25	77.9
5	45	24	45	77.0
6	55	24	45	77.9
7	45	72	45	84.1
8	55	72	45	83.1
9	42	48	35	75.3
10	58	48	35	77.0
11	50	8	35	74.4
12	50	88	35	86.7
13	50	48	18	72.1
14	50	48	52	86.0
15	50	48	35	85.2

Table 4
ANOVA results for variance analysis of the regression model

Source	Sum of squares	Degree of freedom (*df*)	Mean square	*F*-value	(Probability>F) *P*-value
Model	463.09	9	51.45	25.87	<0.0001
X_1	0.021	1	0.021	0.011	0.9194
X_2	137.20	1	137.20	68.99	<0.0001
X_3	115.85	1	115.85	58.26	<0.0001
X_1X_2	1.13	1	1.13	0.57	0.4693
X_1X_3	1.28	1	1.28	0.64	0.4410
X_2X_3	0.50	1	0.50	0.25	0.6269
X_1^2	141.89	1	141.89	71.35	<0.0001
X_2^2	36.08	1	36.08	18.14	0.0017
X_3^2	64.31	1	64.31	32.34	0.0002
Lack of fit	19.89	5	3.98		
Pure error	0.000	5	0.000		
Corrected total	482.98	19			

The experimental data were analyzed by Design Expert (Version 7.1). The adequacy of the developed model and statistical significance of the regression coefficients were tested using the analysis of variance (ANOVA). Eq. 4 is the quadratic model equation relating the cellulose conversion ratio to the tested independent variables in terms of coded variables:

$$Yc=-268.686+12.413X_1+0.516X_2+1.320X_3-0.003X_1X_2+0.008X_1X_3+0.001X_2X_3-0.126X_1^2-0.003X_2^2-0.021X_3^2 \quad (4)$$

The ANOVA was used to test the statistical significance of the response surface quadratic model and the results were listed in Table 4. Fisher's *F* value (25.87) with a low probability value ($p<0.0001$) indicated the high significance of the model. A fairly high R^2 (0.9588) implied that the regression model was statistically significant and only 0.0412% of the total variations were not explained by the model. Therefore, the model was applicable to predict the cellulose conversion yield within the limits of the experimental factors.

Optimization using desirability functions

To obtain the optimized parameters, the partial derivative of functions of variables (*i.e.*X_1, X_2 and X_3)with respect to *Y* was conducted on Eq. 4, which thenled to Eq. 5:

$$\frac{\partial Y}{\partial X_1}=0;\frac{\partial Y}{\partial X_2}=0;\frac{\partial Y}{\partial X_3}=0 \quad (5)$$

Therefore, the optimal conditions were found as follows: X_1=50°C, X_2=48 h, X_3=35 FPU/g, and the calculated cellulose conversion ratio was 85.2%. An independent experimental test was conducted under the conditions predicted by the model. An average value of the cellulose conversion ratio of 85.4% was obtained, indicating the reliability of the RSM modes.

Contour plots of three independent variables

In order to study the interaction among the different independent variables and their corresponding effect on the response, the contour plots are presented in Figure 1 (a, b, c). As can be seen, the range of the optimum temperature was narrow for a high cellulose conversion ratio and 50°C was preferable. Figure 1b clearly shows that the cellulose conversion ratio was greatly affected by the interaction effect of enzyme loading and treatment temperature. This behavior suggests that the cellulose conversion was greatly enhanced due to the high enzyme loading under the optimum temperature. The cellulose conversion ratio was improved with an extended treatment time and an increased enzyme loading at 50°C. The enzymatic hydrolysis is a heterogeneous reaction, which includes cellulase adsorption, formation of substrate-cellulase complexes, and saccharide dissolution, hence a certain time is required. Moreover, the temperature is the main factor to accelerate the protein-based cellulase activity. Similarly, Zhu *et al.*[21] used cellulase to hydrolyze sulfite pretreated pine under the conditions of 50 °C, 15 FPU/g and 48 h, and obtained 79.9% of total glucose, xylose and mannose recovery.

Characterization of enzymatic hydrolysate

The enzymatic hydrolysis liquor obtained under

the optimum conditions was analyzed by HPLC and the results are listed in Table 5. As can be seen, the glucose was the primary component in the hydrolysis liquor, while xylose concentration was quite low. Other degradation material was not detected, indicating the compatibility with the fermentation process.

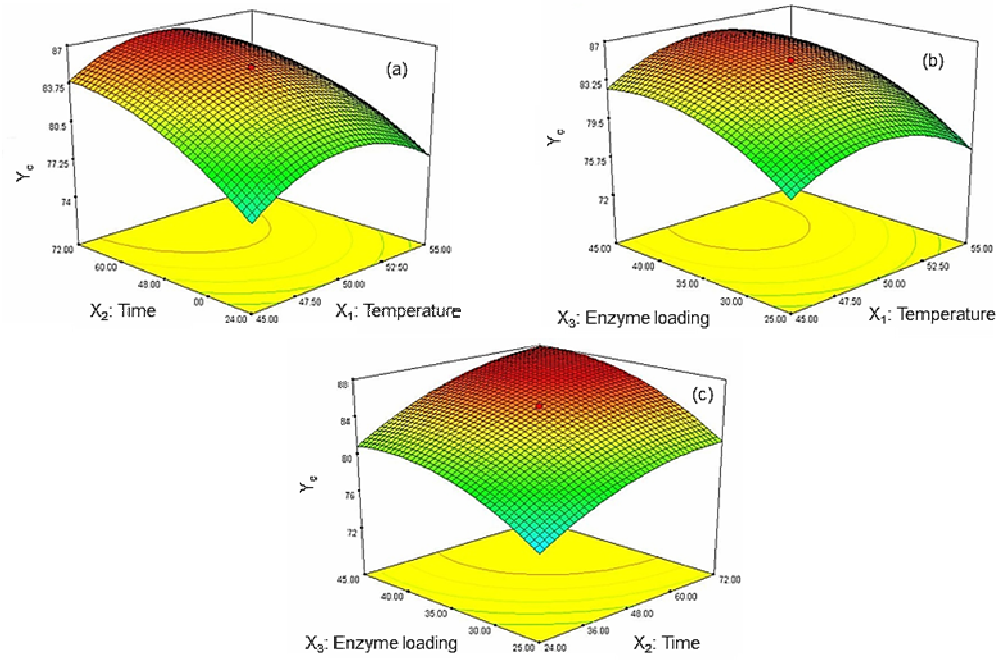

Figure 1: Contour plot of temperature and time (a), temperature and enzyme loading (b), and time and enzyme loading (c) on cellulose conversion ratio

Table 5
HPLC analysis of enzymatic hydrolysis liquor

Composition	Glucose	Xylose	Arabinose	Xylitol	Glycerol
Concentration(g/L)	13.4	0.3	0	0	0

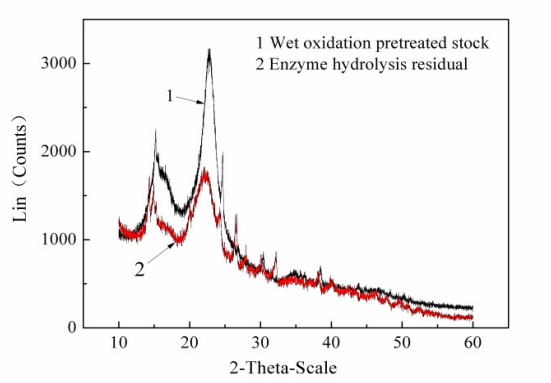

Figure 2: X-RD of WO pretreated substrate and enzymatic hydrolysis residue

X-RD and SEM of enzymatic hydrolysis residue

Figure 2 shows the X-RD spectra of the WO pretreated substrate and of the enzymatic hydrolysis residue. The crystallinity of the WO pretreated

substrate and enzyme hydrolysis residue was 65.5% and 36.2%, respectively, indicating that the crystalline region and the amorphous region were digested in the enzymatic hydrolysis process. However, Hall *et al*. observed that the crystallinity of the enzymatically hydrolyzed substrate stayed constant (around 60%) during glucose production from pure Avicel cellulose, by using CP/MAS ^{13}C-NMR analysis.[22]

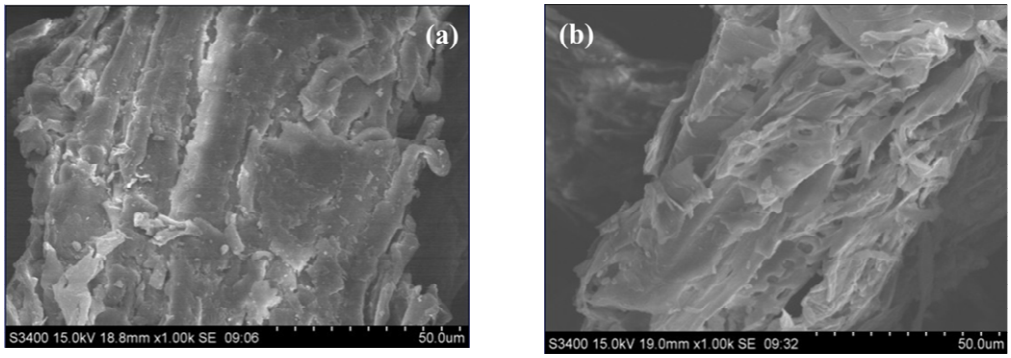

Figure 3: SEM of WO pretreated substrate (a) and enzymatic hydrolysis residue (b)

Figure 3 shows the SEM of the WO pretreated substrate and of the enzymatic hydrolysis residue. It can be noted that there are cracks on the surface of pretreated substrate, indicating the degradation and dissolution of lignocellulose during the WO pretreatment. After enzymatic hydrolysis, many more holes and gaps appeared on the surface of the residue, which was the result of the cellulase hydrolysis and dissolved cellulose.

CONCLUSION

The conditionsfor the enzymatic hydrolysis of wet oxidation pretreated wood pulp waste were optimized using response surface methodology (RSM). An empirical quadratic model equation was developed for the enzymatic hydrolysis process, which was verified by the independent experimental results. The optimum conditions were found as follows: temperature of 50°C, time of 48 h and enzyme loading of 35 FPU/g substrate. X-RD and SEM analyses of the enzymatic hydrolysis residue showed that the cellulose was degraded to a great extent during the enzymatic hydrolysis of the WO substrate.

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