Simulation and Optimization on Cellulase Immobilization Using Response Surface Methodology

Q. Zhang, Y. Lin, S. Shen, Z. Xing, and X. Ruan

***Abstract*—To enhance the recovery rateofcellulase immobilization, various factors (enzyme dosage, temperature, pH and glutaraldehyde concentration) that affect the immobilization process were investigated and evaluated using response surface methodology. The exact effect of each factor was successfully simulated through a Box-Behnken design. The results showed these factors had significant liner, quadratic and interactive effects on recovery rate (p<0.05). The predicted optimal condition for this immobilization was an enzyme dosage (chitosan-to-enzyme ratio) 9.3, a temperature of 30.6 ℃, a pH value of 5.3 and aConcentration of glutaraldehyde of 0.14% (m/V). The validation experiment showed the recovery rate of cellulase in this condition was 68.5%, which was in accordance with the predicted value 68.3%. The immobilized cellulase was recycled and reused in the cellulose hydrolysis process for five times and reached over 85% of the free enzyme hydrolysis efficiency.**

***Index Terms*—Cellulase immobilization, cellulose hydrolysis, Recovery rate, response surface methodology.**

I. Introduction

Enzyme hydrolysis is one of the vital processes of the biofuel production from lignocellulose such as straw, which is to convert cellulose to reducing sugar for the subsequent ethanol fermentation [1]. High price of cellulase is the main cost in the bioethanol production, which greatly restricts the industrialization of bioethanol producing [2]. Therefore, it is necessary to recycle and reuse of the enzyme after the hydrolysis reaction by means of immobilizing enzyme onto carrier.

Several factors may influence the enzyme's performance in hydrolysis, such as pH, temperature, dosage of carrier or enzyme, etc. A useful statistical technique for the modelling and optimisation of complex immobilization processes, Response surface methodology (RSM), was adopted to regulate and optimize the immobilization of cellulase, compared to the traditional "one factor at a time" methodology, RSM could interpret and analyse the combined influence of the parameters affecting immobilization efficiency, and furthermore predict the optimal immobilization condition [3].

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II. Methods

A. Materials for Immobolization

Chitosan, Poly-(1,4-b-D-glucopyranosamine), was selected as the carrier for the immobilization of cellulase, while glutaraldehyde acted as the cross-linking agent. The commercial cellulase from *Trichodermaviride* that was used for the immobilization onto chitosan was purchased from KAYON, Shanghai, China. The filter paper activity (FPA) of this cellulase was 5.09 FPU/g.

B. Preparations for the Carriers

2% (m/v) chitosan was dissolved in 4% (m/v) acetic acid solution by ultrasonic method, and then the solution was dropped into the solidification solution through syringe with a 0.7 mm needle to form the beads. The solidification solution consisted of 30% (v/v) ethanol and 10% (m/v) sodium hydroxide. The carriers were leached carefully and then collected through suction filtration to remove the water after 30 minutes' standing. The beads should be stored under 4 °C [4].

C. Immobilization of Cellulase

The solid cellulase was immobilized onto the carriers through absorbing-crosslinking. Carriers beads soaked in citratebuffer with a certain pH controlled over night before the immobilization process. Cellulase was added into the buffer and the solution was vibrated for 4 hours under 180 r/min and a consistent temperature for the cellulose protein to be absorbed on the surface of carriers. Then, the cross linking agent glutaraldehyde was added to the solution and the solution was vibrated again under the same condition [5].

D. Evaluation of the Immobilization Performance

The performance of immobilization could partially be evaluated by immobilized efficiency (*IE*), which was calculated according to (1),

$$IE = (m_1\text{-}m_2)/m_1 \times 100\% \quad (1)$$

where m_1 stands for the total mass of enzyme protein added, and m_2 stands for the mass of enzyme protein in the leachate. The mass of protein was tested through Bradford method.

The standard recovery rate (*RR*) of immobilized cellulase was calculated according to (2),

$$RR = EA_t/EA_0 \times IE \times 100\% \quad (2)$$

where EA_0 and EA_t stand for the enzyme activity of cellulase before and after immobilization, respectively. The enzyme activity of free and immobilized cellulase was both measured

as Measurement of cellulose activities, offered by Laboratory Analytical Procedure (LAP) [6].

E. Experimental Design and Analysis

TABLE I: INDEPENDENT VARIABLES AND LEVELS IN THE BOX-BEHNKEN DESIGN

Independent variables	symbols	Range and levels		
		-1	0	1
Enzyme dosage ($m_{carriers}$: m_{enzyme})	X_1	2.0:1	6.0:1	10.0:1
Temperature (℃)	X_2	20.0	30.0	40.0
pH	X_3	4.0	5.0	6.0
Concentration of glutaraldehyde (% m/V)	X_4	0.05	0.17	0.30

RSM was adopted to model and optimize the immobilization process of cellulase. Box-Behnken design (BBD) was used to analyse the effects of the four variables (pH, temperature, enzyme dosage and concentration of glutaraldehyde) on the responses RR. Each variable was set three levels, i.e. -1, 0, and +1. The experiments were designed using Design Expert software version 8.0.6 (Statease, Inc., Minneapolis, MN, USA). The designed levels of the parameters were shown as Table I.

Totally 29 runs of experiments was carried out according to the list from the software .Effects of the variables on RR were simulated based on the obtained numerical data and then predict the optimal immobilization condition [3]. The RSM model for analysing and predicting is as (3),

$$Y = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_{ii} X_i^2 + \sum_{i=1}^{4} \sum_{j=i+1}^{4} \beta_{ij} X_i X_j \quad (3)$$

where Y is the response (or its transformation),X_i is the variable, β_0,β_i, β_{ii}, and β_{ij} are respectively the coefficients of the constant, liner, quadratic and interaction terms of the regression model.

III. RESULTS AND DISCUSSION

A. RSM Results on RR

The results of the RSM experiments were analysed by Design Expert 8.0.6. The mathematical quadratic model (insignificant terms were eliminated) relating the RR to the independent variables, is shown below,

$$(RR+0.2)^{-0.2}= -0.053X_1-0.00613X_2+0.027X_3+0.012X_4-0.07X_1X_3 -0.014X_1X_4+0.01X_3X_4+0.045X_1^2+0.018X_2^2+0.049X_3^2 \quad (4)$$

Analysis of variance of the quadratic model was used to evaluate the impact and significance of each term (linear terms, squared terms and interactions) in the regression equation, which was demonstrated in Table II.

The Model F-value of 8.49 implies the model is highly significant (p=0.0001). There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. The R^2 value was 0.8947, which implies that only 2.10% of the total variation was not explained by the model. In additional to the data shown above, the adjusted R^2 was 0.8894, which was in good agreement with the R^2 value. The vicinity of the adjusted R^2 to R^2 indicates a good adjustment of the theoretical response values to the experimental data by the developed model. Adequate precision is reflected in the signal-to-noise ratio, and a ratio greater than 4 is desirable. Furthermore, the adequate precision value was12.436, which is an adequate signal. The low value obtained for the coefficient of variation (2.40 %) indicated the experiments were precise and reliable. Therefore, the developed model was accurate and can be used to navigate the designed space and predict the response of RR.

TABLE II:ANALYSIS OF VARIANCE OF THE QUADRATIC MODEL

Source	Sum of squares	Degree of freedom	Mean square	F value	p-value (Prob>F)
Model	0.091	14	6.48E-03	8.49	0.0001
X_1	0.034	1	0.034	44.95	< 0.0001
X_2	4.51E-04	1	4.51E-04	0.59	0.4546
X_3	8.69E-03	1	8.69E-03	11.39	0.0045
X_4	1.87E-03	1	1.87E-03	2.45	0.1396
X_1X_2	4.12E-07	1	4.12E-07	5.40E-04	0.9818
X_1X_3	0.019	1	0.019	25.49	0.0002
X_1X_4	8.12E-04	1	8.12E-04	1.06	0.3198
X_2X_3	4.83E-05	1	4.83E-05	0.063	0.805
X_2X_4	3.11E-04	1	3.11E-04	0.41	0.5337
X_3X_4	4.01E-04	1	4.01E-04	0.53	0.4806
X_1^2	0.013	1	0.013	16.89	0.0011
X_2^2	2.21E-03	1	2.21E-03	2.9	0.1105
X_3^2	0.015	1	0.015	20.22	0.0005
X_4^2	3.22E-04	1	3.22E-04	0.42	0.5267
Residual	0.011	14	7.63E-04		
Pure Error	4.22E-04	4	1.05E-04		
Cor Total	0.1	28			

B. Effects of Variables on RR

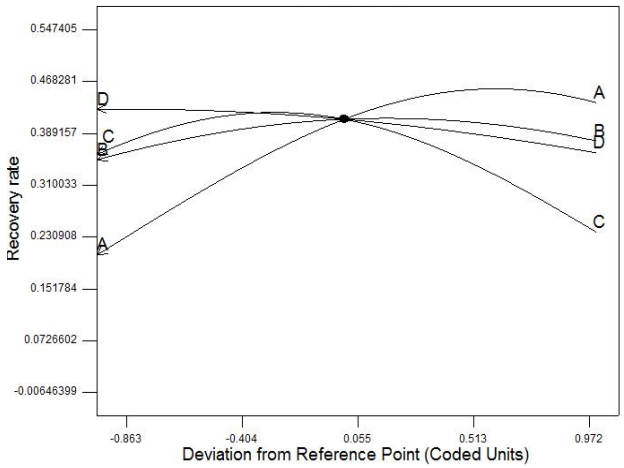

Fig. 1. Perturbation of factors' effect on RRReference point: chitosan-to-enzyme ratio 6, temperature 30 ℃, pH 6.0 and glutaraldehyde concentration 0.17%.

Values of "Prob> F" less than 0.0500 indicate model terms are highly significant, while values greater than 0.1000 indicate the model terms are not sosignificant. The results showed that the enzyme dosage had a significant negative liner effect (p<0.0001) and positive quadratic effect (p=0.0011) on the RR transformation $(RR+0.2)^{-0.2}$, while pH had a significant positive liner and quadratic effect (p=0.0045, 0.0005). According to Fig. 1, RR would be obviously improved with the increase of enzyme dosage and

pH, but stopped increasing, even began decreasing at a certain variable level. And the other two variables did not have such an obvious effect on RR.

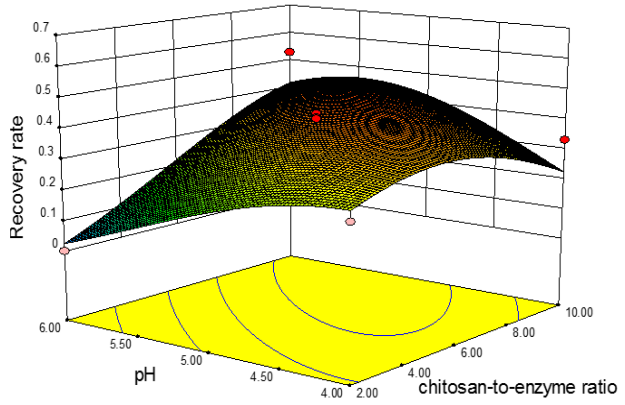

Fig. 2. Interactive effects of enzyme dosage (chitosan-to-enzyme ratio) and pH on RROther parameters: pH 6.0 andglutaraldehyde concentration 0.17%.

Fig. 2 depicted the interactive effect of enzyme dosage and pH on RR. As inferred from Fig. 2, RR increased with an increase in enzyme dosage (chitosan-to-enzyme ratio) until a certain value, meaning a further increase in theenzyme dosagewas deleterious to RR. This trend verified the enzyme dosage had a negative linear effect but a positive quadratic effect on the RR at the 5 % confidence interval. High chitosan-to-enzyme ratio would greatly improve IE since more carriers offered sufficient absorptive sites to cellulase, and thus ensure a higher RR [7]. However, over high ratio of chitosan would lower opportunity for the cellulase's contact with cellulose, resulting a decrease in enzyme activity [3], [8]. Similar results of pH could also be seen in Fig. 1. Too high or too low pH values were detrimental to the enzyme activity, because the proper pH ensured the correct spatial structure of protein [9]. The interactive effect on RR between enzyme dosage and pH was very significant (p=0.0002), since pH determined the enzyme activity of cellulase and the enzyme dosage affected the enzyme concentration as well as the enzyme's contact with cellulose [10]. At a comparatively low chitosan-to-enzyme ratio, RR decreased with the increase of pH, while at a higher enzyme dosage, as the increase of pH, RR firstly increased and then decreased, and the change amplitude was more acute, indicating pH had a more evident effect on RR at a high chitosan-to-enzyme ratio. When pH was maintained at a low level, the RR firstly increased and then descended with the increase of enzyme dosage. By contrast, RR would increase constantly with the rise of enzyme dosage at a higher pH. The contour plot demonstrated the RR reached a relatively high value if the chitosan-to-enzyme ratio was over 5 and the pH was over 4.5.

C. Optimization of Cellulase Immobilization

The model predicted a maximum RR value of 68.3 % at the optimal conditions of enzyme dosage (chitosan-to-enzyme ratio) 9.3, a temperature of 30.6 ℃, a pH value of 5.3 and aglutaraldehyde concentration of 0.14% (m/V). The verificationtests showed the immobilized beads had an IE of 99.1%, an enzyme activity of 3.52 FPU/g, and a RR value of 68.5%. The properties of the immobilized enzyme were highly coordinate to the prediction, which proved the accuracy of RSM. And through the optimization, the RR was improved from 52.3% (the highest value got in the 29 runs of experiment design) to 68.5%. The RSM was well applied in regulation and optimization of cellulase immobilization just as W. Zhang's research in Optimization of simultaneous saccharification and fermentation using the same method [3].

The immobilized enzyme proved to be recycled and reused for five times, and the average hydrolysis efficiency could reach over 85% of the free cellulase under the same hydrolysis condition (solid straw concentration 4% (m/v), equivalent enzyme amount 26.7 FPU per gram of straw, pH 5.4, stirring rate 200 r/min, reaction time 96 h). It could be calculated that over 70% of the enzyme could be saved after five cycles of hydrolysis. The reuse and recycle of the expensive enzyme was realized and thus the production cost of fuel ethanol from lignocellulose was successfully reduced.

IV. Conclusion

The immobilization process of cellulase was successfully simulated through RSM and the comprehensive influence of different variables on RR was detected. A well-fitted regression equation with an R^2 value of 0.8947 of this mathematical model was obtained. The optimal condition for the cellulose immobilization was an enzyme dosage (chitosan-to-enzyme ratio) 9.3, a temperature of 30.6 ℃, a pH value of 5.3 and aConcentration of glutaraldehyde of 0.14% (m/V). The predicted RR at this condition was 68.3%, and the validation experiment proved it to be 68.5%, which was in accordance with the predicted value. The Box-Behnken design and the developed model can be used to navigate the designed space and predict the immobilization process. Moreover, the immobilized cellulase was successfully recycled and reused for five times for cellulose hydrolysis, and exceeded 85% of the free enzyme efficiency.

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