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Optimization of the Medium for the Production of Cellulase by the Mutant *Trichoderma reesei* WX-112 Using Response Surface Methodology

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Summary

The mutant strain *Trichoderma reesei* WX-112 with high cellulase activity was isolated by a newly invented plate. The mutant's ability to produce cellulase increased 1.95 times after the treatment with UV and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). Also, the medium composition was optimized using response surface methodology (RSM). A fractional factorial design (2^{6-2}) was applied to elucidate the medium components that significantly affect cellulase production. The concentration of Avicel and soybean cake flour in the medium were significant factors. The steepest ascent method was used to locate the optimal domain and a central composite design was used to estimate the quadratic response surface from which the factor levels for maximum production of cellulase were determined. The composition of fermentation medium optimized with response surface methodology was (in g/L): wheat bran 30, Avicel 36.4, soybean cake flour 24.7, KH_2PO_4 4 and corn steep flour 5. Compared to the original medium, the cellulase activity increased from 7.2 to 10.6 IU/mL.

Key words: cellulase, *Trichoderma reesei* WX-112, optimization, fractional factorial design (FFD), central composition design (CCD), response surface methodology (RSM)

Introduction

Cellulosic material is the most abundant renewable carbon source in the world. Cellulose may be hydrolyzed using enzymes to produce glucose, which can be used for the production of ethanol (*1*), organic acids (*2*), and other chemicals (*3*). An important impediment in the exploitation of cellulose is the fact that the production of cellulase is expensive, contributing as much as 50 % to the overall cost of hydrolysis (*4*). This is due to the low specific activity of cellulase, necessitating a large quantity of the enzyme for extensive hydrolysis. Considerable progress has been made in strain development (*5, 6*), optimization of culture conditions (*7–9*), and mode of cultivation (*10–13*). High cellulase production has usually been obtained in fed-batch and continuous cultivation using a fermenter (*11,14*). However, the use of crystalline cellulose as insoluble substrate in a culture system gives rise to difficulties in transferring the cellulosic material in continuous or fed-batch culture. Also, studies have demonstrated that the application of soluble sugar as substrate does not result in high cellulase productivity (*15*).

The optimization of fermentation conditions is an important problem in the development of economically feasible bioprocesses. Combinatorial interactions of me-

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dium components with the production of the desired compound are numerous and the optimum processes may be developed using an effective experimental design procedure. Response surface methodology (RSM), which is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors and searching for the optimum conditions, has successfully been used in the optimization of bioprocesses (*16–20*).

The objective of this work was to apply RSM to evaluate the effects of the medium components on cellulase production by the mutant *T. reesei* WX-112 and to search for the optimal medium composition for attaining a higher cellulase yield.

Materials and Methods

Microorganisms

T. reesei RUT C-30 was obtained from the Culture Collection Center of Southern Yangtze University. The strain of *T. reesei* WX-112, an active producer of cellulase, is a mutant derived from *T. reesei* RUT C-30. It was maintained on slants of potato dextrose agar (PDA) and subcultured every month.

Production of mutants

The parent strain (*T. reesei* RUT C-30), with high cellulase activity, was subjected to mutagenesis with UV, and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) as reported previously (*5*). The irradiated spores were plated out on the cellulose agar surface and incubated at 28 °C for 2 to 3 days until clear zones around three to four colonies were observed. The colonies with large zones of clearance were subcultured on PDA and tested for the production of cellulase in shake flasks.

Plate-clearing assay

A kind of very simple and highly efficient plate invented by the authors was used to screen for hypercellulase-producing mutants. The basic medium consists of ball milled cellulose and 2 % agar. A concentration of 0.5 % L-sorbose was added to this plate because it had been reported that L-sorbose could inhibit the mycelium growth and induce the production of cellulase (*21*). The plates were seeded with the irradiated spores and incubated at 28 °C for only 2 to 3 days, after which clear zones could be observed only around colonies of the mutant strains. This is due to the more immediate hydrolysis of the cellulose in the vicinity of the colonies of the mutant strains, as a result of the greater cellulase production by these colonies.

Cellulase production

For the preparation of inoculum, 5.0 mL of a spore suspension (containing 10^8 conidia/mL) of *T. reesei* WX--112 was inoculated into 100 mL of the seed medium containing (in g/L): wheat bran 15, Avicel 10, soybean cake flour 10, KH_2PO_4 3, corn steep flour 5, $CaCl_2$ 0.3 and $MgSO_4{\cdot}7H_2O$ 0.3, in a 250-mL conical flask, the initial pH of the medium was adjusted to pH=5.4 by citrate buffer, and cultured at 30 °C and 180 rpm for two days. Small scale experiments were carried out in 500-mL conical flasks with 100 mL of fermentation medium. The inoculum volume ratio was 10 %, and the flasks were shaken at 150 rpm and 29 °C for 7 days.

Enzyme assay

Filter paper activity (FPA) and carboxymethyl cellulose (CMC) activity were determined according to the method of the International Union of Pure and Applied Chemistry (IUPAC) and expressed as international units (IU). One international unit of cellulase activity is the amount of enzyme that forms 1 μmol glucose (reducing sugars as glucose) per minute during the hydrolysis reaction. Reducing sugar was determined by the dinitrosalicylic acid (DNS) method (*22*).

Experimental design and optimization

RSM consists of a group of empirical techniques devoted to the evaluation of relations existing between a cluster of controlled experimental factors and the measured responses, according to one or more selected criteria (*23–25*). A prior knowledge and understanding of the process and the process variables under investigation are necessary for achieving a more realistic model. A 2^{6-2} fractional factorial designs (FFD) was used to pick factors that influence cellulase production significantly and insignificant ones were eliminated in order to obtain a smaller, more manageable set of factors. In FFD, the range and the levels of the variables investigated in this study are given in Table 1. The central values (zero level) chosen for experimental design were (in g/L): wheat bran 30, Avicel 25, soybean cake flour 20, KH_2PO_4 4, yeast extract (YE) 10 and corn steep flour 5. In develop-

Table 1. Factors and coded values of FFD

Variables /(g/L)	Symbol		Range and levels		
	Real	Coded	–1	0	1
wheat bran	Z_1	X_1	20	30	40
Avicel	Z_2	X_2	15	25	35
soybean cake flour	Z_3	X_3	10	20	30
KH_2PO_4	Z_4	X_4	2	4	6
YE	Z_5	X_5	5	10	15
corn steep flour	Z_6	X_6	2	5	8

ing the regression equation, the test variables were coded according to the equation:

$$X_j = (Z_j - Z_{oj}) / \Delta_j \qquad /1/$$

where X_j is the coded value of the independent variable, Z_j is the real value of the independent variable, Z_{oj} is the real value of the independent variable on the centre point and Δ_j is the step change value. The linear model obtained is expressed as follows:

$$y = \beta_0 + \sum_{j=1}^{3} \beta_j X_j \qquad /2/$$

where y is the predicted response, X_j are input variables which influence the response variable y; β_0 is the intercept; β_j is the jth linear coefficient.

If the mean of the center points exceeds the mean of the factorial points, the optimum would be near or within the experimental design space. If the mean of the center points was less than the mean of the factorial points, the optimum would be outside the experimental design space and the method of the steepest ascent should be applied. The direction of the steepest ascent is parallel to the normal contour line of response curve of the model (Eq. 1) and passes through the center point of FFD. Increment is direct ratio to regression coefficients β_j. Experiments were performed along the steepest ascent path until the response did not increase any more. This point would be near the optimal point and can be used as center point to optimize the medium.

Once critical factors were identified via screening and significant gross curvature was detected in the design space, the central composite design was proceeded to obtain a quadratic model, consisting of trials plus a star configuration to estimate quadratic effects and central points to estimate the pure process variability and reassess gross curvature, with cellulase production as response. For two factors, the model obtained was expressed as follows:

$$y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_{11}^2 + \beta_{22} X_{22}^2 + \beta_{12} X_{12} \qquad /3/$$

where y is the measured response, β_0 is the intercept term, β_1 and β_2 are linear coefficients, β_{12} is the interactive coefficient, β_{11} and β_{22} are quadratic coefficients, and X_1 and X_2 are coded independent variables. Low and high factor settings are coded as –1 and 1, the midpoint is coded as 0. The factor settings of trails that ran along axes drawn from the middle of the cube through the centers of each face of the tube are coded as 1.414 or –1.414. The experimental design and results are presented in Table 2. The SAS software, version 8.0 was used for regression and graphical analyses of the data obtained. The optimal concentrations of the critical medium components were obtained by ridge analysis and also by analysing the contour plots. The statistical analysis of the model was performed in the form of analysis of variance (ANOVA).

Table 2. Design and results of central composition design (CCD)

Run	X_2	X_3	FPA / (IU/mL)
1	–1	–1	7.9
2	–1	1	6.4
3	1	–1	7.1
4	1	1	6.5
5	–1.41	0	9.1
6	1.41	0	6.1
7	0	–1	8.0
8	0	1.41	6.3
9	0	0	10.6
10	0	0	10.6
11	0	0	10.4
12	0	0	10.5
13	0	0	10.2

Results and Discussion

Obtaining the mutants

The mutation program was begun with UV light and MNNG individually and in combination as mutagens. The combination of them proved to be successful and a cellulase hyperproducing mutant was isolated and named as *T. reesei* WX-112, whose ability to produce cellulase increased 1.95 times compared with the parent strain. The screening procedure of the cellulase-producing mutant strains is as follows:

T. reesei RUT C-30 (3.7 IU/mL) $\xrightarrow{\text{UV light}}$ *T. reesei* RUT UV-15 (4.5 IU/mL) $\xrightarrow{\text{MNNG}}$ *T. reesei* RUT NT-36 (5.9 IU/mL) $\xrightarrow{\text{MNNG}}$ *T. reesei* RUT WX-112 (7.2 IU/mL)

Fractional factorial design

According to the results above and from primary studies (*7*,*11*), Avicel (X_2) and soybean cake flour (X_3) were selected as the main carbon and nitrogen source, adding wheat bran (X_1), KH_2PO_4 (X_4), YE (X_5) and corn steep flour (X_6) to optimize the medium composition using 2^{6-2} fractional factorial design. Coded values of factors, design and results of experiment were shown in Tables 1 and 3.

The factorial analysis of variance in Table 4 indicated that the concentration of Avicel (X_2) and soybean cake flour (X_3) are significant factors (p value of <0.05 was used as a cutoff point for significant differences) affecting cellulase production of *T. reesei* WX-112, while wheat bran, KH_2PO_4, YE and corn steep flour were considered non-significant ones. As YE has no significant effect on cellulase production and its price is high, it was eliminated from the medium. Interactions among variables were not significant. A linear regression equation could be obtained from the regression results of fractional factorial experiment:

$$y = 7.588 - 0.147 X_1 + 0.823 X_2 + 0.366 X_3 + 0.119 X_4 + 0.079 X_5 + 0.203 X_6 \qquad /4/$$

The regression coefficients and determination coefficient (R^2) for the linear regression model of cellulase production are presented in Table 4. The model was highly significant ($p<0.01$) and adj.R^2=0.941. The significant difference between the mean (7.6 IU/mL) of responses at

Table 3. Experimental design and results of FFD

Run	X_1	X_2	X_3	X_4	X_5	X_6	FPA/(IU/mL)
1	–1	–1	–1	–1	–1	–1	6.0
2	1	–1	–1	–1	1	–1	6.1
3	–1	1	–1	–1	1	1	7.8
4	1	1	–1	–1	–1	1	8.4
5	–1	–1	1	–1	1	1	7.2
6	1	–1	1	–1	–1	1	7.4
7	–1	1	1	–1	–1	–1	9.0
8	1	1	1	–1	1	–1	7.9
9	–1	–1	–1	1	–1	1	6.5
10	1	–1	–1	1	1	1	6.9
11	–1	1	–1	1	1	–1	8.6
12	1	1	–1	1	–1	–1	7.5
13	–1	–1	1	1	1	–1	7.8
14	1	–1	1	1	–1	–1	6.3
15	–1	1	1	1	–1	1	9.0
16	1	1	1	1	1	1	9.1
17	0	0	0	0	0	0	7.3
18	0	0	0	0	0	0	7.1
19	0	0	0	0	0	0	7.2
20	0	0	0	0	0	0	7.2

Table 4. Regression results of FFD

Term	Estimate	Pr > |t|
Intercept	7.588	0.0001
X_1	–0.147	0.2734
X_2	0.823	0.0001
X_3	0.366	0.0175
X_4	0.119	0.3678
X_5	0.079	0.5441
X_6	0.203	0.1411

Table 5. Results of the steepest ascent path experiment

Run	Z_2	Z_3	FPA/(IU/mL)
Origin	25	20	7.2
1	27.5	21.1	7.8
2	30	22.2	8.6
3	32.5	23.3	9.2
4	35	24.4	10.2
5	37.5	25.6	10.6
6	40	26.7	10.3
7	42.5	27.8	9.4
8	45	28.9	8.6

all fractional factorial points and the response (7.2 IU/mL) at the center points indicated that the optimal point is outside the experimental design space and the method of steepest ascent should be applied.

Steepest ascent path

The direction of the steepest ascent path can be determined by Eq. 4 and the regression results. Since the concentration of wheat bran, KH_2PO_4, and corn steep flour were insignificant factors, their concentrations were fixed at zero level. Avicel (X_2) and soybean cake flour (X_3) were significant factors, and coefficients of X_2 and X_3 were positive, which means that increasing their concentrations has positive effects on the cellulase production. Avicel was chosen as a standard because its coefficient is higher. One basal increment (D) was defined as the increase of Avicel concentration of 2.5 g/L each time. Experimental design of the steepest ascent and corresponding results are shown in Table 5. After the fifth step on the path, further experimentation cannot increase the cellulase activity. The highest enzyme activity was achieved in the fifth step. These results indicate that the concentration of Avicel and soybean cake flour of the fifth step was near optimal, thus the fifth step was chosen as the center point to optimize the medium composition.

Central composite design

Concentration of Avicel (Z_2 =37.5 g/L) and soybean cake flour (Z_3=25.6 g/L) in the fifth step were chosen as the center point to optimize the medium composition with a central composite design. Table 2 shows the design of this experiment and the results. Regression analysis was performed to fit the response function with the experimental data. The statistical significance of the second-order model equation was checked by an F-test (ANOVA) and the data are shown in Table 6. The regression model for cellulase production was highly significant ($p<0.01$) with a satisfactory value of determina-

Table 6. ANOVA results for cellulase production obtained from CCD

Regression	DF	Sum of Squares	R^2	F-value	Pr > F
Linear	2	5.490	0.1335	10.06	0.0087
Quadratic	2	33.477	0.8142	61.35	0.0001
Crossproduct	1	0.240	0.0058	0.88	0.3794
Total model	5	39.206	0.9535	28.74	0.0002

tion coefficient (R^2=0.95), indicating that 95 % of the variability in the response could be explained by the second-order model equation given below (Eq. 5):

$$y = 10.46 - 0.60\ X_2 - 0.57\ X_3 - 1.53\ X^2_{22} - 1.76\ X^2_{33} + 0.25\ X_2X_3 \qquad /5/$$

The ANOVA results showed that this model is appropriate. The test also suggested that the production by *T. reesei* WX-112 was primarily determined by the linear and quadratic terms of Avicel and soybean cake flour of the model and no significant interaction existed between the two factors.

The resulting response surface showed the effect of Avicel and soybean cake flour concentration on the cellulase production (Fig. 1). This result demonstrated that the response surface had a maximum point. The maximum production of cellulase by *T. reesei* WX-112 was obtained in the optimized medium when the initial concentration of Avicel and soybean cake flour was 36.4 and 24.7 g/L, respectively. The maximum response predicted from the model was 10.7 U/mL. Repeated experiments were performed to verify the predicted optimum. The result from three replications (*i.e.* 10.5, 10.7 and 10.6 U/mL) was coincident with the predicted value and the model was proven to be adequate. The final concentration of the medium optimized with RSM was (in g/L): wheat bran 30, Avicel 36.4, soybean cake flour 24.7, KH_2PO_4 4 and corn steep flour 5. Compared with the original medium, the cellulase activity increased from 7.2 to 10.6 IU/mL.

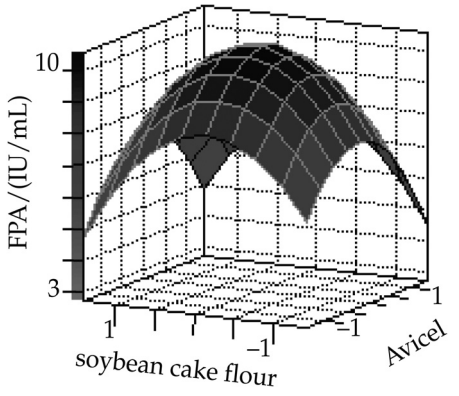

Fig. 1. Response surface plot for the effect of Avicel and soybean cake flour on cellulase production

Conclusions

The results strongly support the mutant selection by the invented plate and the use of RSM for medium optimization. The mutation and the optimization of the medium resulted not only in increasing FPA up to 10.6 IU/mL but also in reducing the cost of the medium. The invented plate for screening mutants and the chosen method of the optimization of medium composition were efficient, relatively simple and time and material saving.

Recent developments in biochemistry, genetics and protein, as well as in the structure–function relationships of cellulases from bacteria and fungi, have led to speculation and anticipation of their enormous commercial potential in biotechnology and research. However, to meet the growing demand for cellulases and to realize their full potential in biotechnology and research, continued multidisciplinary research on basic and applied aspects is vital.

Our work on cellulase production will be continued with experiments on fed-batch and repeated fed-batch fermentations, aiming at higher yields and productivities. The yield is rather important parameter, as the cost of raw material constitutes a large part of the total production cost. It is also important to produce fermentation broths with high enzyme activities in order to use the cellulases in a commercial hydrolysis process without too much dilution of the hydrolysate. The data obtained in this and future studies will be used to model the process and make economical evaluations of the ethanol production process.

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Optimiranje podloge za proizvodnju celulaze s pomoću mutiranog soja *Trichoderma reesei* WX-112 metodom odzivnih površina

Sažetak

Mutirani soj *Trichoderma reesei* WX-112, velike celulazne aktivnosti, izoliran je na tek izumljenoj ploči za uzgoj. Sposobnost mutiranoga soja da proizvodi celulazu povećala se 1,95 puta nakon obrade UV-zračenjem i *N*-metil-*N'*-nitro-*N*-nitrozogvanidinom (MNNG). Sastav podloge optimiran je metodom odzivnih površina. Reducirani faktorski plan (2^{6-2}) primijenjen je kako bi se objasnio značajan utjecaj komponenata na proizvodnju celulaze, a osobito koncentracija Avicela i odmašćenog sojinog brašna u podlozi. Metodom najstrmijeg uspona utvrđeno je optimalno područje, a centralno uređenim planom određena je kvadratna odzivna površina prema kojoj se vide razine faktora za maksimalnu proizvodnju celulaze. Podloga optimirana metodom odzivnih površina sadržavala je 30 g/L pšeničnih mekinja, 36,4 g/L Avicela, 24,7 g/L odmašćenog sojinog brašna, 4 g/L KH_2PO_4 i 5 g/L kukuruzne mokre meljave. U usporedbi s početnom podlogom celulazna se aktivnost povećala od 7,2 na 10,6 IU/mL.