A Shortcut to the Optimization of Cellulase Production Using the Mutant *Trichoderma reesei* YC-108

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Abstract *Trichoderma reesei* YC-108, a strain isolated by a kind of newly invented plate was found to over produce cellulase and it was then used as a cellulase producer. To get the maximum amount of cellulase, the combination of the medium ingredients, which has a profound influence on metabolic pathway was optimized using response surface methodology. The optimum composition was found to be 24.63 g/L wheat bran, 30.78 g/L avicel, and 19.16 g/L soya-bean cake powder. By using the optimized medium, the filter paper activity (FPA) increased nearly five times to 15.82 IU/mL in a 30 L stirred fermenter, carboxymethyl cellulase activity (CMCase) was increased from 83.02 to 628.05 IU/mL and the CMCase/FPA ratio was nearly doubled compared with the parent strain at initial medium.

Keywords *Trichoderma reesei* YC-108 · Cellulase · RSM · Optimization

Nomenclature

X_i, X_j Independent variable coded value
 Z_j Independent variable real value, g/L
 X_l Coded value of wheat bran concentration

X_2 Coded value of avicel concentration
 X_3 Coded value of soya-bean cake powder concentration
 Y Predicted response (cellulase activity), IU/mL

Greek Symbols

b_0 Intercept term
 b_j Linear effect coefficient
 b_{ij} Interaction effect coefficient
 b_{jj} Squared effect coefficient
 Δ_j Step change value

Introduction

Cellulose is a renewable carbon source that is available in large quantities, and can be a solution to the problems of ethanol [1], organic acids [2], and other chemicals [3]. The major problem is cost factor of exploitation of cellulase, with contribute as much as 50 % to the overall cost [4]. In order to improve the yields of cellulase, lots of work has been made in developing new strains, optimizing culture conditions [5–8], and cultivation mode [9–11].

In the present work, strain *Trichoderma reesei* RUT C-30 was conducted by using a combination of UV light and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG), a cellulase hyperproducing strain *T. reesei* YC-108 was obtained, whose CMCA/FPA ratio is much higher than parent strain. It is helpful to improve the application value. In process of optimizing the medium ingredients, we found that one kind of nitrogen source, soya-bean cake powder, could enhance cellulase activity significantly. And then we optimized the three most important medium ingredients using response surface methodology (RSM). To our knowledge, it has not yet been reported to study and optimize conditions of a

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medium for cellulase production. Finally, the process was scaled up in a 30 L stirred fermenter.

Materials and Methods

Microorganisms

Strain *T. reesei* RUT C-30 was purchased from China centre of industrial culture collection (CICC); *T. reesei* YC-108, a mutant derived from *T. reesei* RUT C-30, was an active producer of cellulase.

Plate-clearing Assay

A kind of convenient and high efficiency screening plate invented by the authors was used to screen for hyper cellulase-producing mutants. The basic medium consists of 2 % cellulose specially treated by homogenizing in a ball mill for 15 min and 2 % agar. Especially, 0.5 % L-sorbose with effect of inhibiting mycelium growth and inducing production of cellulase was added to this plate [12]. The plates were seeded with irradiated spores and incubated at 28 °C for 2–3 days, a clear zones would be observed only around colonies of the mutant strains.

Cellulase Production

For inoculum preparation, 5.0 mL of a spore suspension (containing 10^8 conidia/mL) of *T. reesei* YC-108 was inoculated into 100 mL of the seed medium in a 250 mL conical flask, and cultured at 30 °C, 180 rpm for 2 days. Small scale experiments were carried out in 500 mL conical flasks with 100 mL of fermentation medium. The inoculum ratio was 10 % (v/v), and the flasks were shaken at 29 °C, 150 rpm for 7 days.

A 30 L stirred-tank fermenter with a working volume of 22 L was used for large scale production of cellulase. The fermentation temperature was 28–30 °C, the agitation speed and air flow rate were kept at 150 rpm and 0.8 vvm, respectively. During the whole fermentation process, samples were periodically withdrawn for detection.

Enzyme Assay

Filter paper activity (FPA) and carboxymethyl cellulose (CMC) activity of the produced cellulase were determined according to the method of the International Union of Pure and Applied Chemistry (IUPAC) [13]. Filter paper was from Xinhua company, and the degree of substitution (DS) and polymerization (DP) of CMC from Noviant Inc used in the study were 0.65 and 750, respectively.

Experimental Design and Optimization

Response surface methodology 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 [14–16]. A prior knowledge and understanding of the process and the process variables under investigation are necessary for achieving a more realistic model [17]. The production medium contained wheat bran, avicel, soya-bean cake powder, potassium (KH_2PO_4), magnesium ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) and trace metals. The significant independent variables of the medium components are wheat bran and avicel [8]. During our previous studies, we also tried many other cheap carbon and nitrogen sources. We found that soya-bean cake powder is a very efficient nitrogen source which can enhance the production of cellulase significantly. So we also select soya-bean cake powder as an significant independent variable. The concentration of other components was kept constant throughout the investigation since they had no significant effect on cellulase production.

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 (g/L): wheat bran = 25, avicel = 30, soya-bean cake powder = 20. In developing the regression equation, the test variables were coded according to the equation

$$X_j = (Z_j - Z_{0j})/\Delta_j, \quad (1)$$

where X_j is the independent variable coded value, Z_j is the independent variable real value, Z_{0j} is the independent variable real value on the centre point and Δ_j is the step change value. The response variable was fitted by a second order model in order to correlate the response variable to the independent variables. The general form of the second degree polynomial equation is

$$Y = b_0 + \sum_{j=1}^3 b_j X_j + \sum_{i < j}^3 \sum_{i < j}^3 b_{ij} X_i X_j + \sum_{j=1}^3 b_{jj} X_j^2$$

where Y is the predicted response, X_i , X_j are input variables which influence the response variable Y ; b_0 is the intercept;

Table 1 Experimental range and levels of the independent variables

Variables (g/L)	Symbol		Range and levels		
	Real	Coded	–1	0	1
Wheat bran	Z_1	X_1	20	25	30
Avicel	Z_2	X_2	25	30	35
Soya-bean cake powder	Z_3	X_3	15	20	25

b_j is the linear coefficient; b_{jj} is the quadratic coefficient and b_{ij} is the interaction coefficient.

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 contour plots. The statistical analysis of the model was performed in the form of analysis of variance (ANOVA).

Results and Discussion

Obtain the Mutant

After conducted by 254 nm UV light with distance of 15 cm for 3–5 min, the mutant named as UV-5 was isolated with higher cellulase activity. And then the UV-5 was treated by 0.2 mg/mL NTG in pH 8.0 Tris buffer for 0.5–1.5 h, the cellulase activity of the mutant named as NTG-2 increased 1.46 times.

The combination of UV light and NTG was more efficient to get the mutants with increased cellulase activity of 1.16 times than NTG-2. The cellulase hyperproducing mutant was isolated and named as *T. reesei* YC-108.

Optimization of Medium

In order to find the optimum combination of major components of the medium, experiments were performed according to the Box–Behnken experimental plan (Table 2).

In order to check the adequacy of the model to represent the system further, an ANOVA table was constructed (Table 3). The results demonstrate that the model is highly

significant, as is evident from the Fisher's F test (F_{model} , mean square regression/mean square residual = 36.18) with a very low probability value [$(P_{\text{model}} > F) = 0.0005$]. The goodness of fit was checked by determination coefficient ($R^2 = 0.985$) which indicates that only 1.5 % of the total variations are not explained by the model. The value of the adjusted determination coefficient ($\text{Adj } R^2 = 0.9577$) is also very high to advocate for a high significance of the model [17, 18]. A higher value of the correlation coefficient, $R = 0.992$, justifies an excellent correlation between the independent variables [19]. At the same time, a relatively lower value of the coefficient of variation ($\text{CV} = 3.27\%$) indicates a better precision and reliability of the experiments carried out [17, 19].

The application of RSM yielded the following regression equation which is empirical relationship between cellulase activity (Y) and the test variables in coded unit.

$$Y = 10.37 - 0.156X_1 - 0.21X_2 - 0.286X_3 - 0.663X_1X_1 - 0.988X_1X_2 - 0.84X_1X_3 - 0.985X_2X_2 - 1.668X_2X_3 - 1.103X_3X_3$$

where Y is the response and X_1 , X_2 and X_3 are the coded values of the test variables respectively.

The significance of each coefficient was determined by P values which are listed in Table 4. The smaller the P -value, the more significant is the corresponding coefficient [17, 18]. The result suggest that these three components can act as limiting nutrients and a little variation in their concentration will alter the product formation rate.

The 2D contour plots are generally the graphical representations of the regression equation, they are presented in Figs. 1, 2 and 3 from which the values of FPA for different concentrations of the variables can be predicted. Each contour curve represents an infinite number of combinations of two test variables with the other one maintained at its zero level, and the maximum predicted value is indicated by the surface confined in the smallest ellipse in the contour diagram.

Figures 1, 2 and 3 depict that there are significant mutual interactions among these three components. Avicel works as a carbon source for the growth of fungi and an

Table 2 Box–Behnken plan in coded value and observed response (FPA)

Runs	X_1	X_2	X_3	$Y(\text{FPA})$
1	−1	−1	0	8.40
2	−1	1	0	9.59
3	1	−1	0	9.83
4	1	1	0	7.07
5	0	−1	−1	6.87
6	0	−1	1	9.75
7	0	1	−1	10.15
8	0	1	1	6.36
9	−1	0	−1	8.15
10	1	0	−1	9.75
11	−1	0	1	9.14
12	1	0	1	7.38
13	0	0	0	10.39
14	0	0	0	10.46
15	0	0	0	10.26

Table 3 ANOVA for the quadratic model

Source	DF	SS	MS	F value	$P > F$
Model	9	27.531	3.059	36.177	0.0005
Residual	5	0.423	0.085		
Lack of fit	3	0.402	0.134	13.015	
Pure error	2	0.021	0.01		
Total	14	27.954			

DF Degrees of freedom, SS Sum of squares, MS Mean of squares

Table 4 ANOVA for Y(FPA)

Term	Estimate	Std err	<i>t</i>	Pr > <i>t</i>
X_1	−0.156	0.103	−1.520	0.189025
X_2	−0.21	0.103	−2.043	0.096542
X_3	−0.286	0.103	−2.784	0.038703
$X_1 X_1$	−0.663	0.151	−4.378	0.007169
$X_1 X_2$	−0.988	0.145	−6.792	0.001053
$X_1 X_3$	−0.84	0.145	−5.778	0.002185
$X_2 X_2$	−0.985	0.151	−6.509	0.001279
$X_2 X_3$	−1.668	0.145	−11.469	0.0001
$X_3 X_3$	−1.103	0.151	−7.285	0.000762

Fig. 1 Contour plot of FPA (IU/mL): the effect of wheat bran and avicel on cellulase production. The concentration of soya-bean cake powder is held at zero level

inducer for the production of cellulase, when the concentration of it increased to 30 g/L or higher level, a decrease in FPA resulted as shown in Figs. 1 and 3, partly due to the pH decreased to <3.0. Sternberg reported that the loss of cellulase activity due to a decrease in medium pH was not recoverable after the pH was adjusted upward [20]. So the optimum avicel concentration is around 27.1–31.6 g/L (Figs. 1, 3). Wheat bran, on the one hand, is a good substrate for cellulase production, because of its nutrients, starch, proteins, and lignocellulosic materials for microbial growth and cellulase synthesis [21], on the other hand, addition of wheat bran can alleviate pH decrease during growth on avicel and resulted in the increase of cellulase activity. So there is a significant mutual interaction between avicel and wheat bran in accordance with Fig. 1. Soya-bean cake powder firstly used as an medium ingredient for cellulase production could increase cellulase activity remarkably. In addition to acting as a nitrogen

Fig. 2 Contour plot of FPA (IU/mL): the effect of wheat bran and soya-bean cake powder on cellulase production. The concentration of avicel is held at zero level

source, it might also contain some inducers or activators responsible for the biosynthesis of cellulase. There are significant mutual interactions between soya-bean cake powder and other two components, and an optimal concentration of soya-bean cake powder is around 18.3–20.6 g/L (Figs. 2, 3).

Ridge Analysis was used to obtain the optimal values of the test variables, in coded unit they are as follows: $X_1 = -0.075$, $X_2 = 0.078$, $X_3 = -0.168$, with the corresponding $Y = 10.44$ IU/mL. The natural values obtained

Fig. 3 Contour plot of FPA (IU/mL): the effect of avicel and soya-bean cake powder on cellulase production. The concentration of wheat bran is held at zero level

Table 5 Cellulase activity before and after optimization produced by *T. reesei* Rut C-30 and *T. reesei* YC-108

Microorganism	Before optimization			After optimization			In 30 L stirred fermenter after optimization		
	FPA	CMCase	CMCase/FPA	FPA	CMCase	CMCase/FPA	FPA	CMCase	CMCase/FPA
<i>T. reesei</i> Rut C-30	3.24	83.02	25.62	5.60	156.02	27.86	9.68	276.07	28.5
<i>T. reesei</i> YC-108	8.40	289.38	34.45	10.66	385.55	36.18	15.82	628.05	39.70

Fig. 4 The time course of cellulase production by *T. reesei* YC-108 in a 30 L stirred fermenter at the optimum medium

by putting the respective values of X_i in Eq. (1) are (g/L): wheat bran = 24.63, avicel = 30.78 and soya-bean cake powder = 19.16.

The model predicts that the maximum cellulase activity obtained is 10.44 IU/mL (a variation of 9.92–10.96 IU/mL being possible) in confidence range of 95 %. Verification of the results using the optimized medium was accomplished by carrying out shake-flask experiments. The maximum FPA obtained experimentally was found to be 10.66 IU/mL obviously in close agreement with the model prediction. After optimization, the production of cellulase was enhanced nearly five times experimentally (Table 5).

Cellulase Production in a 30 L Fermenter

The process of cellulase production was scaled up in a 30 L stirred fermenter with the optimized medium. The time course and pH value on cellulase production were shown in Fig. 4. Since the dissolved oxygen and mass transfer conditions were improved in the stirred fermenter, the fermentation time was reduced to 5 days, and the maximum FPA, CMCA, the CMCA/FPA ratio reached 16.23, 631.45 IU/mL and 38.91, respectively. The results showed that the cellulase production of *T. reesei* YC-108 was increased at large scale by using this optimized medium.

Conclusions

Three variables, the composition of medium, wheat bran, avicel, and soya-bean cake powder were designed using RSM for FPA. The values of these components to obtain the maximum enzyme activity were found, and scaled up in a 30 L fermenter. The mutation and the optimization of the medium not only resulted in increasing the cellulase activity of the mutant *T. reesei* YC-108 up to nearly fivefolds, and the CMCA/FPA ratio two times, but also reducing the cost of the medium by using simple, cheap and efficient medium ingredients. This study could be one of the effective approaches to reduce the cost of cellulase production.

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References

- Olsson L, Hahn-Hagerdahl B (1996) Fermentation of lignocellulosic hydrolysates for ethanol production. *Enzyme Microb Technol* 18:312–331
- Luo J, Xia LM, Lin JP, Cen PL (1997) Kinetics of simultaneous saccharification and lactic acid fermentation processes. *Biotechnol Prog* 13:762–767
- Adsul MG, Ghule JE, Singh R, Shaikh H, Bastawde KB, Gokhale DV, Varma AJ (2004) Polysaccharides from bagasse: applications in cellulase and xylanase production. *Carbohydr Polym* 57:67–72
- Mandels M (1985) Applications of cellulases. *Biochem Soc Trans* 13:414–416
- Deswal D, Khalsa YP, Kuhad RC (2011) Optimization of cellulase production by a brown rot fungus *Fomitopsis* sp. RCK2010 under solid state fermentation. *Bioresour Technol* 102:6065–6072
- Gattinger LD, Duvnjak Z, Khan AW (1990) The use of canola meal as a substrate for xylanase production by *Trichoderma reesei*. *Appl Microbiol Biotechnol* 33:21–25
- Schafner DW, Toledo RT (1991) Cellulase production by *Trichoderma reesei* when cultured on xylose-based media supplemented with sorbose. *Biotechnol Bioeng* 37:12–16
- Yu XB, Yun HS, Koo Y (1998) Production of cellulase by *Trichoderma reesei* Rut C30 in wheat bran-containing media. *J Microbiol Biotechnol* 8:208–213
- Heck JX, Flôres SH, Hertz PF, Ayub MAZ (2005) Optimization of cellulase-free xylanase activity produced by *Bacillus coagulans* BL69 in solid-state cultivation. *Process Biochem* 40:107–112
- Techapun C, Poosaran N, Watanabe M, Sasaki K (2003) Optimization of aeration and agitation rates to improve cellulase-free

- xylanase production by thermotolerant *Streptomyces* sp. Ab106 and repeated fed-batch cultivation using agricultural waste. *J Biosci Bioeng* 95:298–301
11. Yu XB, Yun HS, Koo Y (1999) Cellulase production in fed-batch culture by *Trichoderma reesei* Rut C30. *J Microbiol Biotechnol* 9:44–49
  12. Wang D, Qu YB, Gao PJ (1995) The mechanism of increasing cellulase biosynthesis rate in *Trichoderma* by L-sorbose. *Acta Mycol Sin* 14:143–147 (in Chinese)
  13. Ghose TK (1987) Measurement of cellulase activities. *Pure Appl Chem* 59:257–268
  14. Akhbari A, Zinatizadeh AAL, Mohammadi P, Irandoust M, Mansouri Y (2011) Process modeling and analysis of biological nutrients removal in an integrated RBC-AS system using response surface methodology. *Chem Eng J* 168:269–279
  15. Marcos JC, Fonseca LP, Ramalho MT, Cabral JMS (2002) Application of surface response analysis to the optimization of penicillin acylase purification in aqueous two-phase systems. *Enzyme Microb Technol* 31:1006–1014
  16. Silva CJSM, Roberto IC (2001) Optimization of xylitol production by *Candida guilliermondii* FTI 20037 using response surface methodology. *Process Biochem* 36:1119–1124
  17. Akhnazarova SL, Kefarov VV (1982) Experiment optimization in chemistry and chemical engineering. Mir Publishers, Moscow
  18. Khuri AI, Cornell JA (1987) Response surfaces: design and analysis. Marcel Dekker, Inc., New York
  19. Box GEP, Hunter WG, Hunter JS (1978) Statistics for experimenters. Wiley, New York
  20. Sternberg D (1976) Production of cellulase by *Trichoderma*. *Biotechnol Bioeng Symp* 6:35–53
  21. Chaabouni SE, Belguith H, Hassairi I (1995) Optimization of cellulase production by *Penicillium occitanis*. *Appl Microbiol Biotechnol* 43:267–269