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Study on the submerged fermentation (SMF) of *termitomyces eurrhizus*

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Abstract: The influence of KH_2PO_4, peptone and brown sugar on *Termitomyces fuliginosu's* mycelium formation was studied with the application of the design of quadratic rotation general combination based on determining the growth curve. A quadratic regression model of biomass to the doses of the above three factors was established. The model fit well and therefore the optimum fermentation condition was obtained. Responses of biomass to the three single factors and to their interactions were discussed. Thus, the highest level of biomass, 21.1 g/L, appeared under the optimized conditions when the initial KH_2PO_4, peptone and brown sugar were 1 g/L, 4 g/L and 72 g/L respectively.

Key words: *Termitomyces fuliginous*, Medium optimization, Mathematical model
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INTRODUCTION

The edible fungus, *Termitomyces eurrhizus*, is very nutritional and delicious (Huang, 1993). Southern Chinese call the fungus chick because it tasted like chicken (Zang, 1981; He, 1995). Its total amount of amino acids is more than 273.9 mg/g, of which the eight necessary amino acids account for 42.41% of the total amino acids. In addition, it contains 0.25 g/L of soluble saccharide, raw protein and Fe, Ca, etc (Zhao, 1997). Its special properties include enhancing stomach digestion, strengthening memory, keeping fresh feeling and curing rheumatism. Its mycelium contains many kinds of amino acids, vitamins and trace elements necessary for the human body.

The objective of the present study is aimed at determining the effects of KH_2PO_4, brown sugar and peptone and their interactions on the cell biomass and finding the optimal conditions for obtaining a preparation with high biomass using the statistical optimization method. Experimental design using current and quadratic and rotational combination was used in the present work to enhance cell biomass.

MATERIALS AND METHODS

1. Strain and media

Termitomyces eurrhizus used in this study was obtained from the Shanghai Academy of Agricultural Science. The stock culture was streaked on potato dextrose agar (PDA, agar 1.5%) slants. Seed culture medium contained: starch 1%, sucrose 0.2%, KH_2PO_4 0.1%, $MgSO_4$ 0.015%. Medium for fermentation contained: KH_2PO_4, peptone, brown sugar, yeast extract 0.3%, $MgSO_4$ 0.015%. All media were autoclaved at 121 ℃ for 30 min before use.

2. Fungal growth

T. eurrhizus was streaked on PDA slants and incubated at 26 ℃ until its mycelium grew up to about 2 cm^2 (Chen, 1983). A mycelial inoculum (about 8 mm^2) of *T. eurrhizus*, following growth on PDA, was inoculated into 50 ml of seed culture medium in 250 ml flask. After being cultured statically for 4 hours, the culture was incubated at 26 ℃ on a rotary shaker at 120 r/min until many white small hypha spheroids formed (Oi, 1982; Zhong, 1991; Fan, 1992). Eight milliliters of freshly grown seed culture was transferred to 100 ml of fermentation medium in 500 ml flask, which was incubated on a rotary shaker at 120 r/min at 26 ℃ for 5 days.

3. Assay of pH and residual sugar

A pH-S meter was used for measuring directly the pH of the broth culture. The method for determination of residual sugar in the supernatant was according to Fan(1992).

4. Quantification of fungal dry weight

Biomass concentration in the replacement cultivation was determined by measuring the dry weight of mycelial samples recovered from aliquots of the culture broth after centrifugation.

5. Experimental design

According to preliminary studies on *T. eurrhizus* fermentation, an orthogonal design of rotated combination of three factors method was selected (Mao, 1986; Li, 1994; Zheng, 2000). Results were analyzed by using DPS software. The variables studied were the concentrations of initial peptone (X_1), initial KH_2PO_4(X_2), and brown sugar (X_3). X_{aj} were coded based on a linear functional between the codified and the actual value according to Eq.(1)

$$X_{aj} = (Z_{1j} + Z_{2j})/2,\ \Delta j = (Z_{2j} - Z_{0j})/2 \quad (1)$$

Where X is the real value of the independent variable; Z is the coded value of the independent variable given by the experimental matrix; X_0 is the real value of the central point. ΔX and ΔZ are the differences between the highest and lowest values of real and coded numbers, respectively. The X and Z values are shown in Table 1.

Table 1 Real and coded (in brackets) values for the variables studied in each type of fermentation

Variable	Real and (coded) values
X_1	0.2(−1.682); 0.28(−1); 0.4(0); 0.48(1); 0.6(1.682)
X_2	0.05(−1.682); 0.07(−1); 0.1(0); 0.13(1); 0.15(1.682)
X_3	4(−1.682); 4.8(−1); 6(0); 7.2(1); 8(1.682)

The responses studied were the effects of the initial concentration of KH_2PO_4, brown sugar and peptone on the cell biomass. The design of a quadratic rotation-general combination is shown in Table 1. Three replicates of the central point allowed calculation of the methods' pure error.

A fully quadratic model containing 10 coefficients was used to describe the responses observed to fit Eq.(1):

$$Y = b_0 + b_1 \times X_1 + b_2 \times X_2 + b_3 \times X_3 + b_4 \times X_1^2 + b_5 \times X_2 + b_6 \times X_3^2 + b_7 \times X_1 \times X_2 + b_8 \times X_1 \times X_3 + b_9 \times X_2 \times X_3$$

Where b was the regression coefficients given by the model. X_1, X_2, X_3 represented KH_2PO_4, brown sugar and peptone respectively.

Independent variables that were found significant at $P<0.05$ in the full model were retained in the reduced models. Variables that were significant ($P<0.05$) with models having $R^2>0.75$ were used to generate contour plots for each factor as a function of two variables while the other was held constant.

RESULTS AND DISCUSSION

1. Determination of growth curve

Application of the results of analysis of the pH, total residual sugar and cell biomass (DCW) yielded the growth curve of *T. eurrhizus* in Fig.1 showing that biomass (DCW) maximized after incubation for 120 hours (5 days), so the fermentation should be stopped after 5 days' growth. During the fermentation, pH changed slightly, and was not similar to that as previously reported by Mao Shisong and Li Xiaoju (Mao, 1986; Li, 1994). The sugar had almost been fully consumed after fermentation for more than 150 hours as shown by the residual sugar curve.

2. Optimization of the fermentation medium

Table 2 shows its matrix structure and results. Responses of cell biomass yield to fermentation conditions showed that the medium compositions had great influence on the cell biomass yield of the fungus.

1) Model fitting

Estimated regression coefficients for each independent variable were obtained by multiple linear regression analysis (Table 3) of responses. According to the results of 20 treatments, a comprehensive regression equation of the ratio of biomass to peptone, KH_2PO_4 and brown sugar was obtained:

Table 2 Experimental matrix and the obtained cell biomass yield from fermentation of *T. eurrhizus*

Run	Peptone (g/100 ml)	KH_2PO_4 (g/100 ml)	Brown sugar (g/100 ml)	Biomass yield (g/100 ml)
1	1	1	1	1.717
2	1	1	−1	1.627
3	1	−1	1	1.599
4	1	−1	−1	1.489
5	−1	1	1	2.075
6	−1	1	−1	1.559
7	−1	−1	1	1.666
8	−1	−1	−1	1.452
9	r	0	0	1.845
10	−r	0	0	1.762
11	0	r	0	2.001
12	0	−r	0	1.182
13	0	0	r	2.158
14	0	0	−r	1.668
15	0	0	0	2.152
16	0	0	0	1.985
17	0	0	0	2.258
18	0	0	0	1.937
19	0	0	0	2.009
20	0	0	0	1.991

$$Y = 2.05868 - 0.01321 \times X_1 + 0.15738 \times X_2 + 0.12843 \times X_3 - 0.11906 \times X_1^2 - 0.18590 \times X_2^2 - 0.07226 \times X_3^2 - 0.03250 \times X_1 \times X_2 - 0.06625 \times X_1 \times X_3 + 0.03525 \times X_2 \times X_3 \quad (2)$$

$X_1=(Z_1-0.4)/0.119$, $X_2=(Z_3-6)/1.189$, $X_3=(Z_2-0.1)/0.030$

Z_1, Z_2, Z_3 are the concentrations of peptone, KH_2PO_4, and brown sugar respectively. Table 3 presents the results of variance.

F-test used for the unfitness of the mathematical model: $F_1 = (SS_{LF}/df_{LF})/(SS_e/df_e) = 1.450$, $F_{0.1}(5, 5) = 3.453$. It could concluded that the unfitness was not significant at 0.1 level because 1.450 was smaller than 3.453. But statistical test of the regression equation showed the regression model was significant at 0.01 level($F_2 = (SS_r/df_r)/(SS_s/df_s) = 5.924$, $F_{0.01}(9, 10) = 4.9423$. It was clear that F_2 was bigger than F0.01(9, 10). In almost all cases, the lack of fit was not significant, which meant that the order of the regression was secondary. So it could be concluded that the experiment data were in good accord with the regression model. Consequently the regression equation could be used to predict the optimization of the fermentation medium.

Table 3 Analysis of variance and regression analysis for the fermentation of *T. eurrhizus*

Source	Sum of squares	Degree of freedom	Mean square	Test statistic	Significant level P
X_1	0.0029	1	0.0029	0.1575	0.69984
X_2	0.4144	1	0.4144	22.3586	0.00081**
X_3	0.2760	1	0.2760	14.8900	0.00317**
X_1^2	0.1984	1	0.1984	11.7321	0.00649**
X_2^2	0.5781	1	0.5781	32.9277	0.00019**
X_3^2	0.0800	1	0.0800	4.9751	0.04980*
X_1X_2	0.0104	1	0.0104	0.5585	(0.47208)
X_1X_3	0.0430	1	0.0430	2.3206	(0.15865)
X_2X_3	0.0122	1	0.0122	0.6570	(0.43649)
Regression	1.2293	9	0.1366	$F_2=5.924$	0.00432**
residual	0.2306	10	0.0231		
Lack of fit	0.1364	5	0.0273	$F_1=1.450$	0.26805
Pure error	1.4599	5	0.0188		
Total		19			

* significant ($P<0.1$); ** significant ($P<0.05$); ( ) not significant

Cell biomass of *T. eurrhizus* was affected (linear and quadratic effects) by the three independent variables. Regression coefficients for the fermentation of *T. eurrhizus* showed that individually, peptone, KH_2PO_4 and brown sugar had linear effects and that the three factors in combination, they had quadratic effects. In fermentation of *T. eurrhizus*, interactions were observed between the three independent variables, but the interactions had no apparent significance.

2) Optimization of the initial concentrations of peptone, KH_2PO_4, brown sugar

The initial concentrations of peptone, KH_2PO_4 and brown sugar were considered to be important factors affecting fermentation of fungi according to previous reports. They were considered independently by keeping the other two factors constant (at zero level). Table 4 gives the obtained derivative regression equation for each factor.

Table 4 The effect of single factor on biomass yield

Model	The optimal value
$Y_1 = 2.05868 - 0.01321X_1 - 0.11906X_1^2$	$X_1 = 0(Z_1 = 0.4$ g/100 ml), $X_2 = 0(Z_2 = 0.1$ g/100 ml) $X_3 = 0(Z_3 = 6$ g/100 ml), $Y_{1max} = 2.059$(g/100 ml)
$Y_2 = 2.05868 + 0.15738X_2 - 0.18590X_2^2$	$X_1 = 0(Z_1 = 0.4$ g/100 ml), $X_2 = 0.5(Z_2 = 0.115$ g/100 ml) $X_3 = 0(Z_3 = 6$ g/100 ml), $Y_{2max} = 2.091$(g/100 ml)
$Y_3 = 2.05868 + 0.12843X_3 - 0.07226X_3^2$	$X_1 = 0(Z_1 = 0.4$ g/100 ml), $X_2 = 0(Z_2 = 0.1$ g/100 ml) $X_3 = 1.0(Z_3 = 7.2$ g/100 ml), $Y_{3max} = 2.115$(g/100 ml)

The derivative regression equation was used to plot the curve of each factor influencing the biomass yield as shown in Fig. 2a, 2b, 2c showing that peptone, KH_2PO_4 and brown sugar had significant effects on the biomass yield, and that they were the limiting substrates.

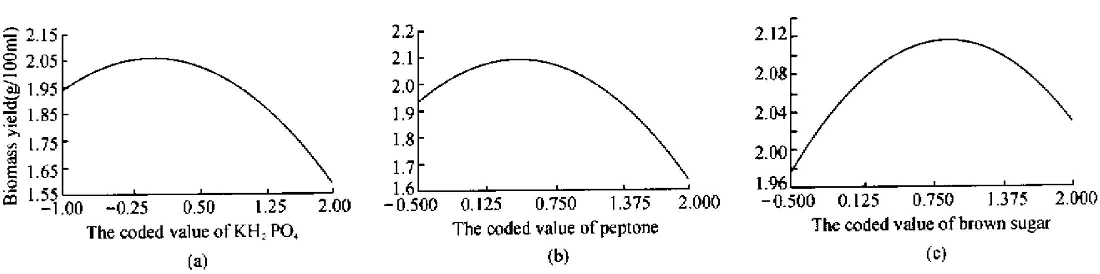

Fig. 2 (a) The influence of peptone concentration on biomass yield; (b) The influence of KH_2PO_4 concentration on biomass yield; (c) The influence of brown sugar concentration on biomass yield

3) Analysis of mutual effect between factors

The model of the relation between the bifactor and biomass was obtained by keeping one of the factor constant (at zero level) (Table 5). Fig. 3a, 3b, 3c present the combined effect of two factors on biomass yield.

The difference between the value of the optimal single factor and that of the optimal bifactor showed the bifactor effect was impor-

Table 5 The effect of two factors on biomass yield

Model	The optimal value
$Y_{12} = 2.05868 - 0.01321X_1 + 0.15738X_2 - 0.11096X_1^2 - 0.18590X_2^2 - 0.03250X_1X_2$	$X_1 = 0(Z_1 = 0.4$ g/100 ml), $X_2 = 0.5(Z_2 = 0.115$ g/100 ml), $X_3 = 0(Z_3 = 6$ g/100 *ml*), $Y_{12max} = 2.0909$(g/100 ml)
$Y_{13} = 2.05868 - 0.01321X_1 + 0.12843X_3 - 0.11906X_1^2 - 0.07226X_3^22 - 0.06625X_1X_3$	$X_1 = 0(Z_1 = 0.4$ g/100 ml), $X_2 = 0(Z_2 = 0.1$ g/100 ml) $X_3 = 1.0(Z_3 = 7.2$ g/100 ml), $Y_{13max} = 2.1149$(g/100 ml)
$Y_{23} = 2.05868 + 0.15738X_2 + 0.12843X_3 - 0.18590X_2^2 - 0.07226X_3^2 + 0.03525X_2X_3$	$X_1 = 0(Z_1 = 0.4$ g/100 ml), $X_2 = 0.5(Z_2 = 0.115$ g/100 ml) $X_3 = 1.0(Z_3 = 7.2$ g/100 ml), $Y_{23max} = 2.1471$(g/100 ml)

tant to biomass yield (Fig. 3a, 3b, 3c). However, the effect was not significant because the central point of each factor was just close to the optimal value, which caused the data to be changed slightly.

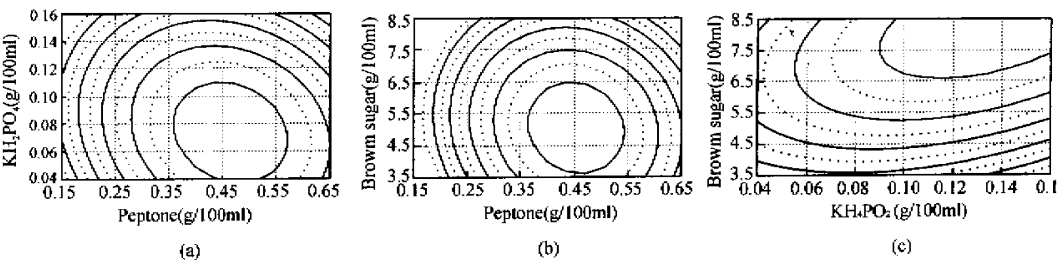

Fig. 3 (a) Contour map of the effect of KH_2PO_4 and peptone on biomass yield; (b) Contour map of the effect of peptone and brown sugar on biomass yield; (c) Contour map of the effect of KH_2PO_4 and brown sugar on biomass yield

Peptone, KH_2PO_4 and brown sugar had significant effect on the biomass yield from the contour curve of bifactors. The biomass yield would decrease a lot when any one of the three factors changed a little near the central point.

CONCLUSIONS

The SMF (submerged fermentation) technique was used in fungus *T. eurrhizus* fermentation to obtain higher biomass yield. By proper selection of initial concentrations of KH_2PO_4, brown sugar and peptone, it was possible to obtain higher biomass yield. Application of the design of quadratic rotated general combination was helpful for determining the optimum medium composition and was proved to be excellent. From the data obtained, it could be concluded that KH_2PO_4, brown sugar and peptone had significant effect on the biomass yield during the fermentation of *T. eurrhizus*. The biomass maximized at 21.1 g/L when the initial concentration of KH_2PO_4, peptone and brown sugar was 0.1%, 0.4%, 7.2%, respectively.

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