

Optimization of Fermentation Medium for Glycyrrhizin Biotransformation to Monoglucuronyl-glycyrrhetic Acid by Plackett-Burman and Box-Behnken Design

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Abstract: Plackett-Burman (PB) and Box-Behnken (BB) experimental designs were used to optimize fermentation variables for the biotransformation of glycyrrhizin (GL) to monoglucuronyl-glycyrrhetic acid (MGGA). The PB design was first used to screen the important factors among the medium variables. The steepest ascent method was used to approach the optimum range for each of these factors. The BB design was finally used to analyze the response surfaces of the screened factors for further optimization. The optimized conditions for this system were 0.7 g/L $MgSO_4 \cdot 7H_2O$, 1.19 g/L GL, and cultivation for six days. The biotransformation of GL to MGGA could reach up to 35.72%, which is a good result for this kind of transformation.

Key words: Glycyrrhizin, *Aspergillus niger*, Plackett-Burman Design, Biotransformation, Box-Behnken Design

1. Introduction

Natural products have been used for thousands of years to promote health and treat diseases, especially in countries such as China, Japan, and Korea. With the development of modern technologies, the exact functions of the components of many of these natural products have been elucidated. For example, the active components of ginseng are ginsenosides, including ginsenosides Rg1, Rg2, Rg3, Rb1, Rb2, and Re. These major components have neuroprotective, cardioprotective, antioxidant, antidiabetic, and anticancer effects [1-3]. Glycyrrhizin, the major component of liquorice root (*Glycyrrhiza glabra*), has been shown to have anti-inflammatory, anticancer, and antiviral properties [4,5]. In ginkgo, the active components are ginkgolides. These components, especially ginkgolide B, present antihypertensive, anti-anaphylactic, and cardioprotective effects [6,7].

In addition to the major components of natural herbs, some rare components have attracted increasing attention because of their physiological effects. For example, taxol and ginsenoside Rh2 are rare components that are effective in cancer treatment [8-11]. For the large-scale preparation of rare components such as ginsenoside Rh2, enzymatic biotransformation may be a feasible method [12-14]. Through hydrolysis of two glucuronides, glycyrrhizin (GL) is transformed to glycyrrhetic acid (GA). If one glucuronide is cleaved, glycyrrhizin is converted to monoglucuronyl-glycyrrhetic acid (MGGA). Because of its moderate polarity, MGGA is more soluble

and more readily transported through the cell membrane than GL and GA. It has been shown that MGGA has a more potent pharmacological effect than GL and GA [15]. With the help of enzymes, major components can be converted to rare components. Although bio-reactions typically require mild reaction conditions and have relatively high selectivity, product yields are still relatively low owing to the complexity of these systems. To optimize complex biotransformation systems, statistical methods such as Plackett-Burman (PB) design and Box-Behnken (BB) design are often used [16,17].

We used PB and BB experimental designs to optimize fermentation variables for the biotransformation of GL to MGGA. Under optimal conditions, the bioconversion may reach 35.72%.

2. Materials and Methods

2-1. Materials

2-1-1. Microorganism

Aspergillus niger was isolated at the Department of Microorganisms of the University of Science and Technology of Liaoning and identified according to its morphology and rDNA gene sequence (CPCCC 480386) by Sangon Biotech (Shanghai) Co. Ltd.

2-1-2. Media

The composition of the slope plate medium was as follows (g/L): potato juice, 200; glucose, 20.00; peptone, 10.00; agar, 20.00 (pH 5.50). The composition of the seed culture medium was as follows (g/L): potato juice, 20.00; raw extract of GL, 1.00; glucose, 10.00; peptone, 10.00 (pH 5.50), and that of the basal fermentation medium was as follows (g/L): 98% GL, 1.20; $NaNO_3$, 1.50; K_2HPO_4 , 1.00; $MgSO_4 \cdot 7H_2O$, 0.50; $FeSO_4$, 0.1; peptone, 1.00 (pH 5.50). All media were sterilized at 121 °C for 20 min.

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2-2. Methods

2-2-1. Preparation of *A. niger* seed culture

Under sterile conditions, an *A. niger* spore suspension was prepared by washing spores from slope plates with sterile water. The suspension was cultivated for 72 h in a rotary shaker at 28 °C and 160 r/min. Under sterile conditions, the seed culture was filtered through gauze and washed several times with sterile water until the prepared mycelial suspension contained no GL, as determined by HPLC. The seed culture was then used for further experiments.

2-2-2. Preparation of fermentation culture

Ten milliliters of seed culture was inoculated into 100 mL of fermentation medium in a 250 mL Erlenmeyer flask and cultured in a rotary shaker at 160 r/min and 28 °C for 120 h. The fermentation culture was centrifuged at 4000 r/min for 10 min and the supernatant was analyzed by HPLC. The percent transformation of GL was calculated with the following formula:

$$A\% = \frac{C_1 V_1}{C_0 V_0} \times 100\%$$

where A is the percent transformation, C_0 is the initial concentration of GL (mg/L) at the start of fermentation, V_0 is the initial volume (mL) of transformation broth, C_1 is the concentration of MGGG (mg/L), and V_1 is the final volume of fermentation medium. Concentrations of GL and MGGG were measured using an Agilent 1100 HPLC system (Agilent Technologies, USA) with an Agilent Extend-C₁₈ column (4.6 mm×150 mm, 5 μm). The following conditions were used for HPLC: mobile phase, 85:15 (v/v) methanol:3.0% acetic acid; column temperature, 25.0 °C; flow rate, 1.00 mL/min; wavelength, 254 nm; UV detector.

2-3. Experimental design

2-3-1. Plackett-Burman design

PB designs, first described by Plackett and Burman [18], are saturated two-level experimental designs based on balanced incomplete blocks. With this method, the main effects can be detected with fewer experimental runs, so important factors influencing an outcome can be identified rapidly and efficiently for subsequent investigation [16].

2-3-2. Steepest ascent method

Once the most influential factors have been identified using PB design, the steepest ascent method can be used to approach the optimal range for each factor, and then effective response surface equations can be established. The steepest ascent method takes the direction of the gradient to be the direction of ascent. The step length is based on the efficient value of various factors. With this method, the optimal range of each influence factor can be determined rapidly and economically.

2-3-3. Box-Behnken design

BB design is a statistical method for solving multivariate problems

that uses multivariate quadratic equations to search the fitting function of influence factors and their response values. The optimization procedure is based on analysis of the regression equation. Compared with other methods used to determine optimal levels of influence factors, such as single-factor experimental and orthogonal designs, BB designs require less experimental time and lead to a more accurate regression equation. BB designs can also be used to explore interactions between several key factors. The method has been widely used to reduce exploration costs, optimize working conditions, and improve product quality in areas such as agriculture, food science, and chemistry [19-20].

3. Results and Discussion

3-1. Estimation of the main influence factors

This fermentation system contained the basic elements required to support *Aspergillus niger* growth, including nutrients such as peptone, nitrogen (applied by NaNO₃), phosphorus, and K (applied by K₂HPO₄) and other inorganic elements such as Na (applied by NaNO₃), magnesium (applied by MgSO₄·7H₂O), and ferrum (applied by FeSO₄). With respect to biotransformation of GL, the concentration of GL was one of the important factors that needed optimization. Generally, the effects of pH and cultivation temperature should be considered in a fermentation process. However, in this work, ten influence factors were investigated: pH (A), temperature (B), GL concentration (C), inoculum size (D), peptone (E), NaNO₃ (F), K₂HPO₄ (G), MgSO₄·7H₂O (H), FeSO₄ (I), and cultivation time (J). Software Design-Expert 8.0.6 was used to design the PB experiment. According to the number of factors in the biotransformation system, the number of experimental runs was set to N=12. Low and high levels of each factor were considered. The response variable used was percent biotransformation, and parallel experiments were carried out. Details of the experimental design as well as the results of the experiment are shown in Table 1. Statistical analysis was performed using SPSS software. The effects of the various factors on the response variable are listed in Table 2, which shows that factors C, H, and J had significant effects (p

Table 1. Plackett-Burman design (coded values) and corresponding response variable measurements (percent transformation; A) (N=12)

Trial number	A	B	C	D	E	F	G	H	I	J	A (%)
1	-1	-1	-1	1	-1	1	1	-1	1	1	27.10
2	1	-1	1	1	1	-1	-1	-1	1	-1	27.24
3	1	1	1	-1	-1	-1	1	-1	1	1	22.49
4	-1	1	1	-1	1	1	1	-1	-1	-1	21.87
5	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	29.14
6	1	-1	-1	-1	1	-1	1	1	-1	1	26.97
7	-1	1	1	1	-1	-1	-1	1	-1	1	28.53
8	-1	1	-1	1	1	1	1	1	1	-1	32.06
9	-1	-1	1	-1	1	1	-1	1	1	1	27.73
10	1	1	-1	1	1	1	-1	-1	-1	1	24.21
11	1	1	-1	-1	-1	1	-1	1	1	-1	34.50
12	1	-1	1	1	-1	1	1	1	-1	-1	29.57

Table 2. Regression coefficients and their significance (Sig.) level

Factor	Effect (B value)	Standard error	t ratio	p-value
Total	36.067	0.989	36.459	0.017*
A	-0.080	0.057	-1.399	0.395*
B	-0.085	0.021	-3.998	0.156*
C	-5.511	0.341	-16.143	0.039*
D	0.067	0.011	5.882	0.107*
E	-18.761	1.707	-10.992	0.058*
F	-1.621	1.138	-1.425	0.390*
G	-18.821	1.707	-11.027	0.058*
H	113.821	4.267	26.674	0.024*
I	180.422	17.068	10.570	0.060*
J	-0.964	0.057	-16.940	0.038*

*Significant at $p < 0.05$

< 0.05). In Table 2, the B value indicates a positive or negative effect of the factor. A positive value for B implies that the corresponding factor should be increased in the next experiment, whereas a negative value implies that the corresponding factor should be decreased. Therefore, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (H) was increased and cultivation time (J) and GL concentration (C) were decreased in the subsequent steepest ascent experiment.

Table 2 shows that magnesium exerts greater effects than do sodium and ferrum. This could be mainly attributed to the fact that magnesium is a necessary trace element for all organisms and plays important roles in physiological activities and, as a cofactor for many enzymes, in energy metabolism, protein and nucleic acid synthesis, and the regulation of cell cycle and cell death.

3-2. Estimation of the levels of optimal factors by the steepest ascent method

Based on the results shown in Table 2, a steepest ascent experiment was conducted. The tested ranges for the most important factors

Table 3. Steepest ascent design and corresponding response values

Trial number	Cultivation time (days)	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (g/L)	GL (g/L)	A (%)
1	7	0.5	1.5	23.357
2	6	0.7	1.25	35.356
3	5	0.9	1	32.672
4	4	1.1	0.75	26.159

GL, glycyrrhizin; A = percent transformation

Table 4. Factors and levels for the Box-Behnken design

Variable No.	Factor	Unit	Lower level (-1)	Fixed Level	Upper level (1)
A	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	g/L	0.6		0.8
B	GL	g/L	1.0		1.5
C	Cultivation time	day	5		7
Fixed	Peptone	g/L		1.0	
Fixed	NaNO_3	g/L		2.0	
Fixed	K_2HPO_4	g/L		1.0	
Fixed	FeSO_4	g/L		0.1	
Fixed	Inoculum size	mL		1.0	
Fixed	Temperature	°C		28.0	
Fixed	pH			5.50	

are shown in Table 3. The other factors were maintained at optimal levels. The corresponding biotransformation results are also shown in Table 3.

As shown in Table 3, the highest percent biotransformation was obtained in trial 2. Therefore, the factor levels from this trial were used as the central point for response surface optimization in the subsequent Box-Behnken experiment.

3-3. Estimation of optimal levels of the main factors by Box-Behnken design

The results of the steepest ascent experiment were used to determine the factors and levels to be tested in the BB design. Details of the design are shown in Table 4.

According to the principle of BB design, three influence factors, each with three levels, were used for the response surface analysis experiment. Two types of experimental data points were selected: the factorial point and the zero point, which was the central point of the investigated range. The results of the 17 trials are shown in Table 5.

3-4. Analysis of BB experimental data

Percent biotransformation was the response variable. Multivariate regression analysis was carried out based on the results shown in Table 5 using the software Design-Expert 8.0.6. The results of the analysis are shown in Table 6. After regression and fitting, a quadratic polynomial regression model was obtained. The response value expressed in terms of variables A, B, and C (coded value) is as follows:

$$A\% = 35.42 + 0.70A - 1.12B - 0.12C - 0.61AB + 0.27AC + 0.062BC - 0.092A^2 - 2.82B^2 - 0.32C^2$$

where A was the dependent variable, the arithmetic mean response of the 17 runs was 35.42, and the estimated coefficients for factors A, B, and C were 0.70, -1.12, and -0.12, respectively. The main effects (A and B) represented the average result of changing one factor at a time from a low to high value. The interactive terms (AB, BC, and AC) show how the response changes when three factors were simultaneously changed. The polynomial terms (A^2 , B^2 , and C^2) were included to investigate nonlinearity. The regression model needed to be evaluated for model significance and the lack-of-fit. Analysis of variance (ANOVA) was used for these evaluations. As shown in Table 6, both linear and quadratic terms of the equation were

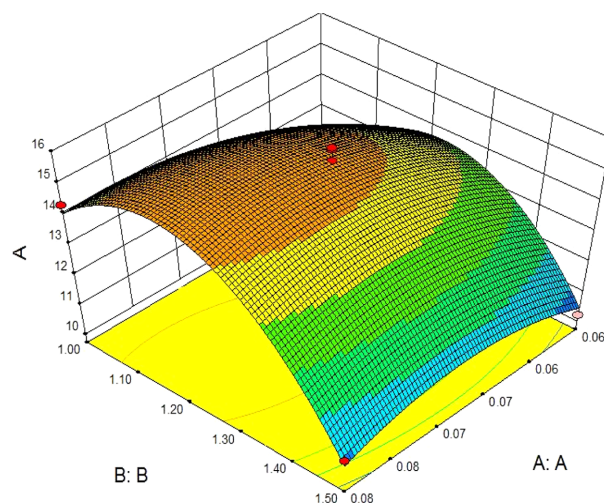
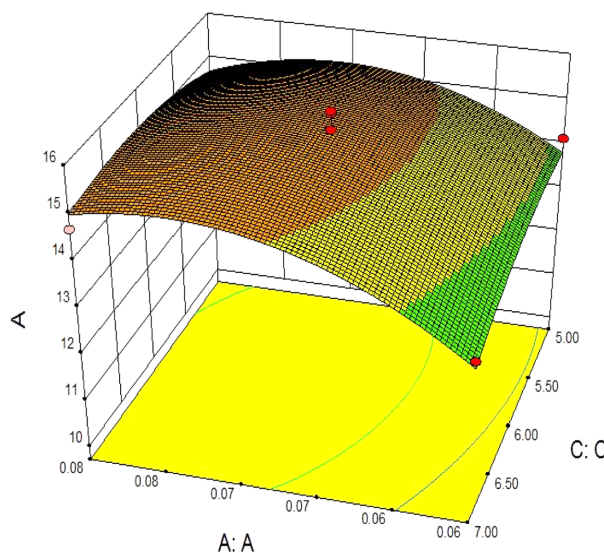
Table 5. Box-Behnken experimental design and corresponding response values (percent transformation; A)

Trial number	A: MgSO ₄ ·7H ₂ O	B: GL	C: cultivation time	A (%)
1	0	-1	-1	33.45
2	1	1	0	30.84
3	0	0	0	35.37
4	0	0	0	34.47
5	0	0	0	35.51
6	0	1	1	31.23
7	-1	0	1	33.22
8	0	0	0	35.90
9	0	0	0	35.84
10	-1	-1	0	31.30
11	0	-1	1	33.26
12	1	0	1	34.72
13	1	-1	0	34.38
14	1	0	-1	34.59
15	0	1	-1	31.18
16	-1	1	0	30.21
17	-1	0	-1	34.17

significant, indicating a nonlinear relationship between the influence variables and percent biotransformation. The coefficient of determination for the regression equation is $R^2 = 0.9688$, indicating that the model is a good fit to the experimental data. The adjusted R^2 indicates that the three influence factors identified in the PB experiment explained 92.88% of the variance in the response variable. In addition, the lack of fit $P > F$ was 0.7167; therefore, the model has practical significance [20,21].

According to the regression equation and the analysis summarized in Table 6, fitted surfaces of each pair of factors in the reaction analysis can be derived; the three-dimensional plots of interactions are shown in Figs. 1-3.

Figs. 1-3 show that the relationship between each pair of factors is parabolic. Fig. 1 shows that GL affected the percent biotransformation more significantly than MgSO₄·7H₂O. The optimum glycyrrhizin concentrations were located between 1.00 and 1.30. As can be

**Fig. 1. Fitted surface for percent transformation as a function of MgSO₄·H₂O (A) and glycyrrhizin (B).****Fig. 2. Fitted surface for percent transformation as a function of MgSO₄·H₂O (A) and cultivation time (C).****Table 6. ANOVA for Box-Behnken design quadratic model**

Source	Sum of squares	df	Mean square	F value	P value	Significance
Model	55.26	9	6.14	24.18	0.0002	Significant
A	3.95	1	3.95	15.56	0.0056	
B	9.96	1	9.96	39.22	0.0004	
C	0.12	1	0.12	0.46	0.5177	
AB	1.51	1	1.51	5.94	0.0450	
AC	0.29	1	0.29	1.14	0.3214	
BC	0.016	1	0.016	0.061	0.8118	
A ²	3.57	1	3.57	14.05	0.0072	
B ²	33.41	1	33.41	131.58	<0.0001	
C ²	0.44	1	0.44	1.72	0.2312	
Residual	1.78	7	0.25			
Lack of fit	0.47	3	0.16	0.47	0.7167	Not significant
Pure error	1.31	4	0.33			
Corrected total	57.04	16				
R^2 0.9688						
Adj. R^2 0.9288						

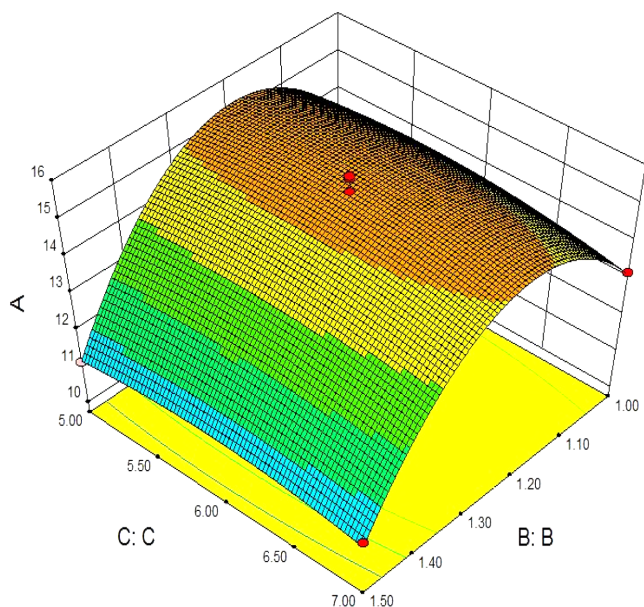


Fig. 3. Fitted surface for percent transformation as a function of cultivation time (B) and glycyrrhizin (C).

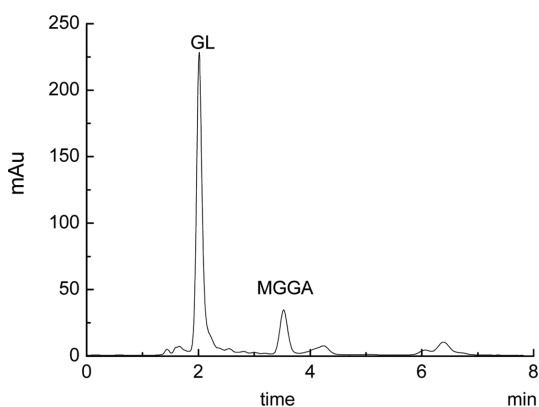


Fig. 4. Chromatogram of the biotransformation of GL. Chromatography condition: column, Extend C₁₈ (4.6 mm×150 mm); mobile phase, methanol and 3.20% acetic acid (85:15, v/v); mobile phase rate, 1.00 ml/min; column temperature 25 °C; UV detector; detective wavelength, 254 nm; injection amount, 10 µl.

seen from Figs. 2 and 3, MgSO₄·7H₂O and GL concentrations affected the percent biotransformation more significantly than cultivation time. The point at which the percent biotransformation was the highest for each factor is indicated in each of the three figures. The optimal conditions derived from the regression equation are 1.19 g/L GL, 0.7 g/L MgSO₄·7H₂O, and cultivation for 6 days. A maximum transformation of 35.72% was obtained under these conditions (Fig. 4). The result obtained is better than that obtained in a previous study [22].

4. Conclusion

Plackett-Burman design was applied to investigate ten fermentation variables to estimate the most important factors influencing the biotransformation of GL to MGGA. The results showed that GL,

MgSO₄·7H₂O, and cultivation time were the most influential factors. Decreasing the cultivation time and the concentration of GL and increasing the concentration of MgSO₄·7H₂O could increase the biotransformation of GL to MGGA. To determine the central point of the response surface in a reasonable and simple way, a steepest ascent experiment was performed to approach the central point. The best fermentation conditions were determined to be 1.00 g/L GL, 0.7 g/L MgSO₄·7H₂O, and six days cultivation time. Subsequently, Box-Behnken (BB) design and response surface analysis were used. A regression model was derived from the BB design and the results showed that there was a maximum point. The optimal fermentation conditions determined by the BB design were 1.19 g/L GL, 2.0 g/L NaNO₃, 0.7 g/L MgSO₄·7H₂O, 1.0 g/L K₂HPO₄, 0.1 g/L FeSO₄, 1.0 g/L peptone, pH 5.50, an inoculum size of 10.0 mL, a temperature of 28.0 °C, and a cultivation time of 6 days, and under these conditions, better percent biotransformation could be achieved.

The application of both Plackett-Burman and Box-Behnken design provided a rapid and effective means to identify the most influential factors for the biotransformation of GL to MGGA. The optimization results fitted very well with the actual fermentation results.

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