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Abstract. The endophytic fungus BS002 with good effect of disease-resistant has been isolated from the medicinal Sophora flavescens. The conditions of the fermentation medium were explored in this paper. On the basis of the single factor experiments, CH3COONa, potato and glucose were defined to be the main factors by Plackett-Burman Design; Response surface methodology (RSM) with Box-Behnke Design was used to optimize fermentation conditions of endophytic fungi BS002. The optimal conditions are defined as follows: 100 mL in the container 250mL, potato 246.47 g/L, glucose 27.81 g/L, CH3COONa 1.95 g/L, concentration of fungus 107 cfu/ml, temperature 25 ℃, yeast extract 1.0 g/L, speed 150 rpm, fermentation for 4 d, dry weight of mycelium can reach 10.398 g/L.

Keywords: response surface methodology; Plackett-Burman; endophytic fungi; fermentation condition; Sophora flavescens

1 Introduction

Endophytic fungi, which lives within healthy plant tissues or organs, do not bring some diseases to plants and the whole or part of the life cycle exists in the plant [1-3]. Studies have shown that, many endophytic fungi were isolated from plant roots, stems, leaves, etc. Part endophytic fungi can produce the same or similar pharmaceutically active ingredients with the host[4,5]. Medicinal plant Sophora with functions of heat-clearing, detoxifying, eyesight-improving, diuretic, rheumatism removing, desinsection, etc. is the dried root of plant, it is cold, bitter, affecting the heart, spleen, kidney. Matrine and flavonoids are contained in Sophora, and have better pharmacologically active and medicinal value[6-9]. Endophytic fungus BS002 strain was isolated from Sophora seeds. The test proved strong antifungal activities against the growth of *Botryosphaeria berengriana f.sp. piricola*, *Physalospora piricola*, *Cladosporium cucumerinum Ell. Arthur.*, *Fusarium oxysporum f.sp. cucumerinum*, *Fusarium moniliforme*, etc. The inhibition to *physalospora piricola* was the biggest with the antibacterial diameter of 45 mm.

In this paper, Plackett-Burman design[10-12] was used to define to the main factors and response surface methodology (RSM) with Box-Behnke design was used to optimize fermentation medium and fermentation conditions[13,14]. RSM, a

mathematical and statistical method, is a way of finding the best conditions in the multi-factor systems. The accurate and effective results can be achieved by the experiment. The best combination of various factors and the optimal response value can be determined by this methed on the entire inspection area[15-19].

2 Experiments and Methods

2.1 Preparation

The endophytic fungus BS002 isolated from the seeds of S. flavescens, was provided by bio-pharmaceutical technology laboratory, College of Biological Engineering, University of Science and Technology LiaoNing, Liaoning Province, China.

Endophytic fungus BS002 (Penicillium sp. M-01, Penicillium variants), was inoculated in PD medium (potato 200 g, sugar 10 g, glucose 10 g, sodium acetate 1.66 g, peptone 1.02 g, water1000 mL) at 25 ℃, 150 r / min training for 4 d, then filtrated.

2.2 Methods of test

Liquid fermentation process parameters with inoculative dose, liquid volume, temperature, Metabolism factor, potatoes, carbon source, nitrogen source, etc. are optimized by single factor experiment. Each factor is set high and low level, denoted by 1 and -1, the high level was 1.5 times of low level in Plackett-Burman Design[19,20](Table 1). The significant factors can be analyzed.

RSM with Design expert 8.0 was used to optimize fermentation conditions of the endophytic fungus BS002. Optimal response can be analyzed and obtained by the significant factors were considered the independent variables and the mycelium dry weight was considered the response value. The design method was showed (Table 2).

Table 1. The measured values of Placket-Burman Design

No.	inoculative dose (cfu/mL)	medium volume (mL)	speed (rpm)	Temper-ature (℃)	CH_3CONa (g/L)	potato (g/L)	glucose (g/L)	yeast extract (g/L)	biomass (g/L)
1	1	-1	+1	-1	-1	-1	+1	+1	5.500
2	1	+1	-1	+1	-1	-1	-1	+1	3.335
3	-1	+1	+1	-1	+1	-1	-1	-1	5.410
4	+1	-1	+1	+1	-1	+1	-1	-1	5.350
5	+1	+1	-1	+1	+1	-1	+1	-1	6.625
6	+1	+1	+1	-1	+1	+1	-1	+1	7.260
7	-1	+1	+1	+1	-1	+1	+1	-1	6.410
8	-1	-1	+1	+1	+1	-1	+1	+1	8.995
9	-1	-1	-1	+1	+1	+1	-1	+1	6.600
10	+1	-1	-1	-1	+1	+1	+1	-1	8.080
11	-1	+1	-1	-1	-1	+1	+1	+1	6.365

12	-1	-1	-1	-1	-1	-1	-1	-1	3.200

Table 2. Level of response surface methodology experiment factors

Level	X_1 : CH_3COONa(g/L)	X_2 : glucose(g/L)	X_3: potato(g/L)
-1	1.5	20	200
0	1.88	25	250
+1	2.25	30	300

The mycelium was collected, washed with deionized water three times, and dried in vacuum oven to constant weight at 150℃ to obtain the biomass of each group(formula 1.).

$$\text{biomass}(g/L) = \text{dry cell weight}(g)/\text{fermentation}(L) \quad \text{(formula 1.)}$$

3 Results and Discussion

3.1 The results of Plackett-Burman Design test

The results of Plackett-Burman Design are shown in Table 3.

Table 3. Levels and effects of variables

NO.	Factors	level		Estimate	t-Value	P-Value	Rank
		-1	1				
1	inoculative dose(cfu/mL)			-0.055	-0.417	0.704	8
2	medium volume(mL)	80	120	-0.010	-1.167	0.328	6
3	speed(rpm)	120	180	0.013	2.374	0.098	4
4	CH_3COONa(g/L)	1.5	2.25	2.847	6.443	0.008	1
5	temperature(℃)	22	33	0.025	0.754	0.505	7
6	potato (g/L)	200	300	0.012	3.521	0.039	3
7	glucose(g/L)	20	30	0.180	5.442	0.012	2
8	yeast extract(g/L)	0.8	1.2	1.242	1.449	0.231	5

According to the Plackett-Burman test, CH_3COONa, potato, glucose were proved to be significant factors. p-values were less than 0.05, and the order: CH_3COONa > glucose > potato. Inoculative dose and medium volume had negative effect on experimental results, that Estimate < 0; the other six fators had positive effect, that

Estimate ＞ 0. Three significant factors would be the basis of the following experiments. According to the single factor experiment results, inoculative dose 10^7 cfu/mL, medium volume 100 mL/250 mL erlenmeyer flask, speed 150 rpm, temperature 25 ℃, yeast extract 1 g/L.

3.2 The results of RSM

The test results are shown in Table 4. and Table 5.

Table 4. Experimental design and result of response surface methodology as N=17

No.	CH_3COONa (g/L)	glucose (g/L)	potato (g/L)	biomass (g/L)
1	1	-1	0	6.898
2	0	-1	1	5.385
3	0	0	0	9.953
4	0	0	0	8.638
5	0	1	1	9.160
6	1	1	0	9.270
7	-1	1	0	8.980
8	0	-1	-1	5.840
9	0	1	-1	8.858
10	-1	0	-1	7.390
11	0	0	0	10.178
12	0	0	0	9.725
13	1	0	1	7.630
14	-1	0	1	7.890
15	0	0	0	9.770
16	-1	-1	0	5.520
17	1	0	-1	9.120

Table 5. Analysis of variance for response surface methodology model

Source	Sum of Square	DF	MeanSquare	F-value	Pr＞F	Significance
Modle	37.71	9	4.19	18.08	0.0005	significaant
A: CH_3COONa	1.23	1	1.23	5.31	0.0546	
B: glucose	19.92	1	19.92	85.95	＜ 0.0001	＊＊
C: potato	0.16	1	0.16	0.7	0.4290	
A.B	0.30	1	0.30	1.28	0.2957	
A.C	0.99	1	0.99	4.270	0.0776	
B.C	0.14	1	0.14	0.62	0.4576	
A^2	1.75	1	1.75	7.55	0.0286	＊
B^2	7.57	1	7.57	32.68	0.0007	＊＊

C^2	4.22	1	4.22	18.19	0.0037	* *
Lack of fit	0.21	3	0.069	0.20	0.8942	not significant
Pure Error	1.41	4	0.35	-	-	
Residuals	1.62	7	0.23	-	-	
Cor Total	39.33	16	-	-	-	
R-Squared 0.9587			AdjR-Squared 0.9057			

Note: * represents significant; ** represents very significant.

The maths model (this test response value Y from the encoded variables A, B, C) was set up based on the above data by quadratic regression analysis with Design expert 8.0.6 : $Y=9.65+0.39A +1.58B - 0.14C-0.27AB-0.50AC+0.19BC-0.64A^2 -1.34B^2- C^2$.

According to analysis of variance, there are significant differences on the biomass between one degree term and quadratic term, in other words, there was no simple linear relationship between experimental factors and response. The model coefficients for each variable are also shown in Table 5. and F-value and P-value were employed to check the significance of each coefficient of the model. Correlation coefficient of the regression equation R^2(R-Squared) =95.87%, it showed that experimental data sufficiently were fitted to the model in the case of significant level $\alpha = 0.01$ and 90.57% data response to the biomass depended on the selected three significant variables. Pr> F term represents the probability is greater than F, lack of fit was 0.8942, not significant, indicating that the equation was adequate for predicting biomass under all conditions, this proved that the model was selected appropriately.

The relationship between the response and experimental data of each variable can be demonstrated by three-dimensional response surface plots which represented the regression equation mentioned above (shown in Fig. 1-4). According to the analysis by the Design-expert 8.0.6, the optimal values of the three key variables for biomass of lipid fermentation of the endophytic fungus BS002 were crystalline sodium acetate 1.95 g/L, glucose 27.81 g/L，potato 246.47 g/L, respectively.

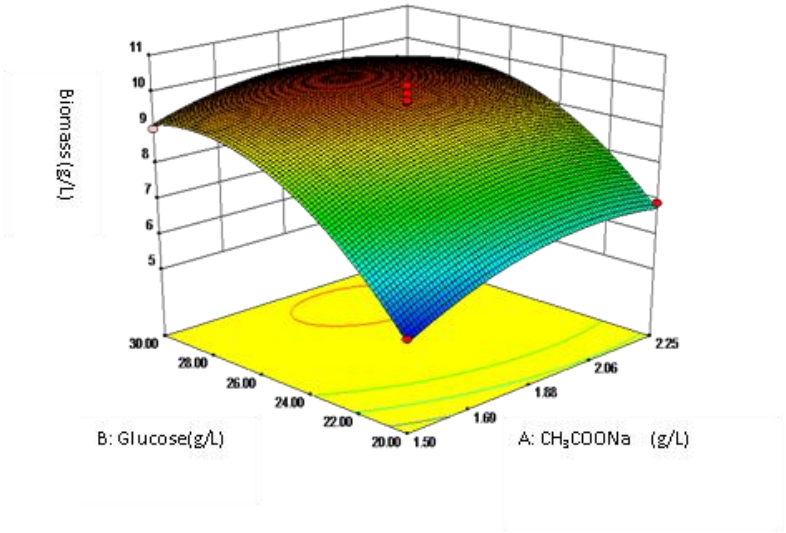

Fig. 1. Response surface figure of regression eqation of biomass VS CH3COONa and glucose

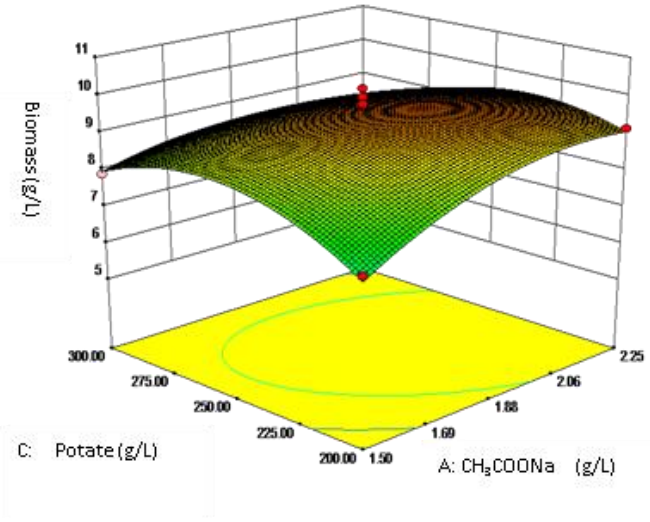

Fig. 2. Response surface figure of regression eqation of biomass VS CH3COONa and potato

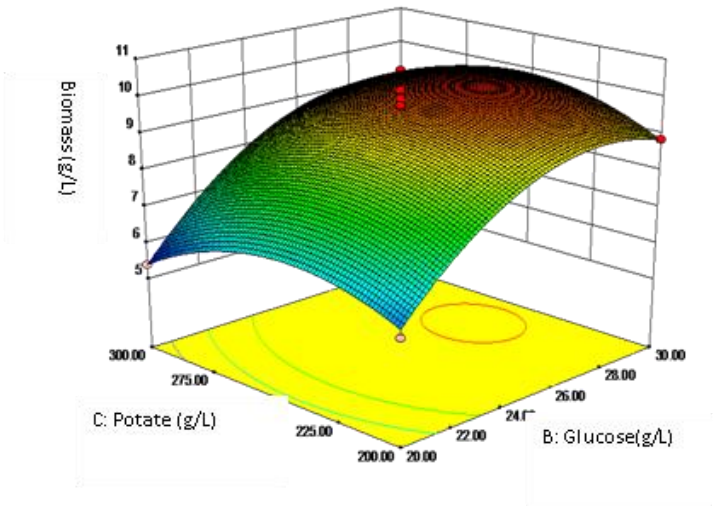

Fig. 3. Response surface figure of regression eqation of biomass VS potato and glucose

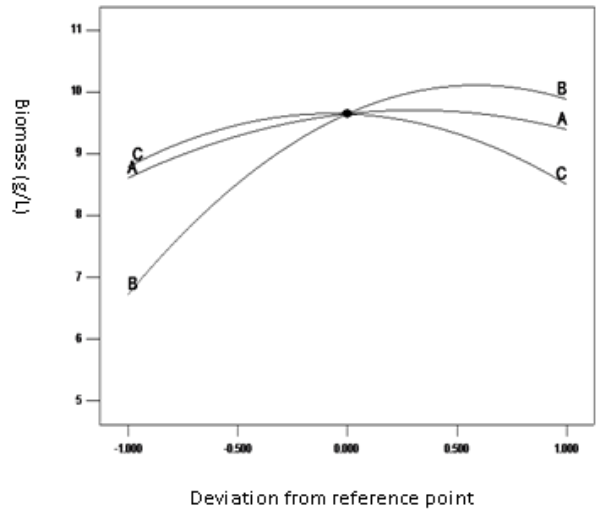

Fig. 4. Binary regression curve of biomass VS concentration of CH_3COONa, potato and glucose

According to the response surface analysis charts, the biomass of endophytic fungus BS002 increased at first and then decreased with the increase in the value of any two parameters.

3 Conclusions

The test by RSM for fermentation condition optimization of endophytic fungus BS002 isolated from *Sophora flavescens* proved that cell dry weight 10.398 g / L on the base of optimized conditions was obtained, and it was increased by 29% compared with 8.062 g / L on the basic of medium, error was only 2.5%, compared to predicted values 10.1429 g / L. It was indicated that the optimal medium combination has important practical applications after the verification test.

Significant effects from liquid fermentation medium composition of endophytic fungus BS002 to cell dry weight were derived in the test. Mathematical model of three significant factors(CH_3COONa, potato, glucose) and biomass was established by RSM using the results of Plackett-Burman Design test. Regression effect of model was significant, it can predict the optimal conditions of the liquid fermentation. The optimal conditions are defined as follows: 100 mL in the container 250 mL, potato 246.47 g/L, glucose 27.81 g/L, CH_3COONa 1.95 g/L, concentration of fungus 10^7 cfu/ml, temperature 25 ℃, yeast extract 1.0 g/L, speed 150 rpm, fermentation for 4 d.

The applications of RSM are gradually widespread at home and abroad, RSM has been extensively applied in the optimization of medium composition, fermentation conditions and food manufacturing processes. It was reported that sulphuric acid-treated sugar cane bagasse hydrolysate can be efficiently used for the cell growth and lipid accumulation of T. fermentans, and it represented a 32.8% improvement in the lipid concentration and a 21.4% increase in the lipid coefficient by RSM[17]. The production of α-amylase by *Aspergillus oryzae* had about 20% increase by RSM for optimizing process parameters[21]. RSM applied in liquid fermentation conditions of endophytic fungus BS002, reduced the workload, and also obtained a better result. A certain foundation to go for separation and purification of the active substance and structure identification is laid.

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