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Full Length Research Paper

Use of response surface methodology to optimize critical medium components for biomass and extracellular polysaccharide production by *Ganoderma sinense*

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Response surface methodology (RSM) was used to optimize the critical medium ingredients of *Ganoderma sinense*. A central composite design (CCD) was employed to determine the maximum biomass and extracellular polysaccharide (EPS) yields at optimum levels for glucose, peptone and KH_2PO_4. A mathematical model was then developed to show the effect of each medium composition and their interactions on the production of mycelial biomass and EPS. The model predicted the maximum biomass yield of 12.58 g/l that appeared at glucose, peptone, KH_2PO_4 of 42.0 5.28 and 0.77 g/l, respectively; while a maximum of EPS yield of 309.6 mg/l appeared at glucose, peptone, KH_2PO_4 of 45.4 4.98 and 0.79 g/l, respectively. These predicted values were also verified by validation experiments. The excellent correlation between predicted and measured values of each model justifies the validity of both the response models. The results of bioreactor fermentation also show that the optimized culture medium enhanced both biomass (13.01 ± 0.36 g/l) and EPS (352 ± 7.9 mg/l) production by *G. sinense* in a large-scale fermentation process.

Key words: *Ganoderma sinense*, submerged culture, response surface methodology, central composite design, extracellular polysaccharide.

INTRODUCTION

Ganoderma is a genus of medicinal polypore mushrooms. Because of their extensive use in traditional Asian medicines, and their potential in bioremediation, they are a very important genus economically. For centries the fungi have been used in traditional Asian medicines for the prevention and treatment of various types of diseases, such as cancer, hepatopathy, arthritis, hypertension, neurasthenia, and chronic hepatitis (Shiao, 2003; Liu and Zhang, 2005). Among the genus, two key species, *Ganoderma lucidum* and *Ganoderma sinense* are indexed in the Pharmacopoeia of the Peoples Republic of China, and used as key medicinal materials of *Ganoderma* (Wang and Li, 2002). Chemistry studies on *G. sinense* and *G. lucidum* have shown that pharmaceutically active compounds from fruiting body and mycelium of *Ganoderma* include polysaccharides, triterpenoids (especially ganoderic acid, GA), steroids, alkaloids, nucleotides, lactones and fatty acids, among which polysaccharides and GAs are the major source of

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biological activity and therapeutic use of *G. lucidum* and *G. sinense* (Shiao, 2003; Sato et al., 2009; Liu et al., 2010; Qiao et al., 2007).

Modern studies show that the polysaccharides from *G. sinense* and *G. lucidum* have various biological functions such as immunomodulating and anticancer effects (Lan et al., 1999; Wang and Li, 2002). The polysaccharides can enhance the immune responses in patients with advanced-stage cancer(Gao et al., 2003), and as a supplement during chemotherapy or radiotherapy, the polysaccharides can enhance the curative effects, reduce side effects such as fatigue, anorexia, hair loss, and bone marrow suppression, etc (Lan et al., 1999; Wang and Li, 2002). Because it usually takes several months to cultivate the fruiting body of *G. sinense* or *G. lucidum* and it is also difficult to control the product quality during its cultivation, there is a great need to supply the market with high-quality of *G. sinense* and *G. lucidum* products. Submerged fermentation of the two fungi is viewed as a promising alternative for the efficient production of mycelial biomass, polysaccharides and ganoderic acid (GA). Currently, most of the research has been related to *G. lucidum*, and to accelerate mycelial growth and metabolite production in *G. lucidum*, the effects of environmental conditions, two-stage culture process, *etc*. have been studied (Yang and Liau, 1998; Fang and Zhong, 2002; Tang et al., 2009; Zhang and Zhong, 2010). In addition, some inducers to increase the mycelial growth and polysaccharide production have been reported (Yang et al., 2000, 2004; Liu and Zhang, 2007). However, data on efficient submerged cultivation of *G. sinense* are scarce, and until now, little attention has been paid to the optimization of media for the production of biomass and polysaccharide by *G. sinense* in submerged fermentation, and the mutual interactions between medium constituents on biomass and polysaccharide production are not well understood.

Response surface methodology (RSM) has been increasingly used for various phases of an optimization process in fermentation (Prapulla et al., 1992; Mao et al., 2005). It is a powerful technique for testing multiple process variables because fewer experimental trials are needed compared to the study of one variable at a time. Also, interactions between variables can be identified and quantified by such technique. In our preliminary experiments (Wang and Liu, 2009), we evaluated the suitability of various carbon sources and nitrogen sources for the effective production of biomass and extracellular polysaccharide (EPS) by *G. sinense*. The preliminary data indicated that the major constituents affecting the performance of culture in terms of biomass and EPS yields were the concentrations of carbon source (glucose), nitrogen source (peptone) and mineral source (KH_2PO_4) (Wang and Liu, 2009).

The objectives of this study were to determine optimal medium components for biomass and EPS production by *G. sinense* and to better understand the relationships between the medium constituents (glucose, peptone and KH_2PO_4) and the biomass and EPS yields.

MATERIALS AND METHODS

Microorganism

The strain of *G. sinense* SCIM 0701 was screened and collected by Strain Collection of Industrial Microorganisms (SCIM), Central South University of Forestry and Technology (Changsha, China). It was maintained on potato-agar-dextrose slant subcultured every 4 weeks.

Flask culture

The culture medium was composed (g/l) of: glucose 35, peptone 5, KH_2PO_4 0.7, $MgSO_4 \cdot 7H_2O$ 0.4, and vitamin B_1 0.01; the initial pH of the medium was adjusted to 6.0. Erlenmeyer flasks (250 ml) containing 50 ml medium were inoculated and incubated at 30℃ in a rotatory shaker at 160 rpm for 8 days to prepare the inoculums. A 10% (v/v) inoculum was added to flask (500 ml) containing 100 ml of medium. The fermentation was incubated at 160 rpm at 30° for 7 days.

Bioreactor fermentation

The bioreactor fermentation was carried out in 30-l mechanical agitated fermenter (made in Jiangsu University, China), under the following conditions: medium volume 20 l, inoculation volume 10% (v/v), temperature 30℃, aeration rate 8.0 vvm, and agitation speed 180 rpm. The fermentation continued until the mycelial biomass and EPS reached their highest values and the residual sugar concentration did not further decrease.

Determination of mycelial biomass and EPS

Culture broth were filtered using 40-mesh stainless sieve and the mycelium was harvested. Biomass was obtained by centrifuging the mycelium at 8000 rpm for 15 min, washing the precipitated cells for three times with distilled water, and drying at 60℃ for sufficient time to a constant weight (Liu and Wang, 2007). For the determination of EPS, after removal of mycelia by centrifugation, the crude EPS was precipitated with addition of 95% (v/v) ethanol by four times of volume, then separated by centrifugation at 10 000 rpm. The insoluble components were measured by phenol–sulfuric acid method (Hsieh et al. 2005; Liu and Wang, 2007).

Measurement of residual sugar

Residual sugar content was determined as 3.5-dinitro-salicylic acid method (Liu and Wang, 2007).

RSM experimental design and statistical analysis

Our preliminary study indicated glucose, peptone and KH_2PO_4 were significant variables for biomass and EPS production. The concentration of $MgSO_4 \cdot 7H_2O$ and V_{B1} were kept constant throughout the investigation since they had no significant effect on biomass and EPS production (Wang and Liu, 2009). A central composite design (CCD) was used in the optimization of biomass and EPS production. The range and the levels of the variables

Table 1. Experimental range and levels of the independent variables.

Independent variables (g/l)	Range and levels				
	-1.682	**-1**	**0**	**1**	**1.682**
Glucose, X_1	18.12	25	35	45	51.82
Peptone, X_2	1.98	3	4.5	6	7.02
KH_2PO_4, X_3	0.33	0.5	0.75	1	1.17

Table 2. The central composite design matrix and the responses of biomass and EPS

Runs	x_1	x_2	x_3	Y_1 Biomass (g/l)	Y_2 EPS (mg/l)
1	-1	-1	-1	5.2	27.2
2	-1	-1	1	5.8	43.1
3	-1	1	-1	6.5	51.3
4	-1	1	1	7.1	65.3
5	1	-1	-1	9.6	137.2
6	1	-1	1	10.4	142.9
7	1	1	-1	12.1	180.7
8	1	1	1	12.9	247.3
9	-1.682	0	0	5.9	22.3
10	1.682	0	0	9.7	329.2
11	0	-1.682	0	6.7	17.9
12	0	1.682	0	10.0	172.1
13	0	0	-1.682	7.7	147.3
14	0	0	1.682	7.9	126.3
15	0	0	0	11.5	234.3
16	0	0	0	11.1	226.3
17	0	0	0	11.2	238.1
18	0	0	0	10.8	235.3
19	0	0	0	10.9	227.7
20	0	0	0	10.6	236.6

investigated in this study are given in Table 1. The lowest and the highest levels of variables were: glucose, 18.12 and 51.82 g/l; peptone 1.98 and 7.02 g/l; KH_2PO_4, 0.33 and 1.17 g/l. A mathematical model, describing the relationships between the process indices (the yield of mycelial biomass and EPS) and the medium component contents in second-order equation, was developed. The yield of mycelial biomass and EPS by *G. sinense* was multiply regressed with respect to the fermentation parameters by the least squares method as follows:

$$Y_i = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \quad (1)$$

Where Y_i is the predicted response variable, β_0, β_i, β_{ii}, β_{ij} are constant regression coefficients of the model, and x_i, x_j (i =1,3; j =1,3, i≠j) represent the independent variables (medium components) in the form of coded values. The accuracy and general ability of the above polynomial model could be evaluated by the coefficient of determination R^2. Table 2 gave central composite design matrix and the responses of biomass and EPS. SAS statistical package (version 8.1, USA) was performed for regression and graphical analysis of data obtained. The optimum concentrations of glucose, peptone and KH_2PO_4 were obtained by solving the regression equation.

RESULTS

Optimization of biomass production

The central composite design and the corresponding experimental data were shown in Table 2. Table 3 shows the analysis of variance for the experiment. The Fisher's F-test with a very low probability value (0.0005) for total model indicated that the model was highly significant, and the coefficient of determination (R^2) was shown as 0.9043, indicating that 90.43% of the variability in the response could be explained by the model. The results of the regression analysis are shown in Table 4. The polynomial model for biomass yield $Y_{biomass}$ was regressed

Table 3. Analysis of variance (ANOVA) for full quadratic model for optimization of biomass production of *G. sinense*.

Regression	DF	Sum of squares	R-square	F value	Pr > F
Linear	3	65.937961	0.6266	21.83	0.0001**
Quadratic	3	28.487844	0.2707	9.43	0.0029**
Crossproduct	3	0.740000	0.0070	0.25	0.8630
Total model	9	95.165805	0.9043	10.50	0.0005**

R^2 = 0.9043; Adj. R^2 = 0.8183; ** Significant at 1 % level.

Table 4. Results of regression analysis of a predictive polynomial model for optimization of biomass production of *G. sinense*.

Factor	Coefficients estimated	t value	Pr > |t|
Intercept	10.987583	26.85	< 0.0001**
x_1	1.9617138	7.907487	0.0001**
x_2	0.9628815	3.881286	0.002183**
x_3	0.2296557	0.925721	0.372834
x_1x_1	-0.947202	-3.92212	0.002027**
x_1x_2	0.300000	0.925537	0.372925
x_2x_2	-0.752746	-3.11693	0.008905**
x_3x_3	-0.947201	-3.92212	0.002027**

** Significant at 1 % level.

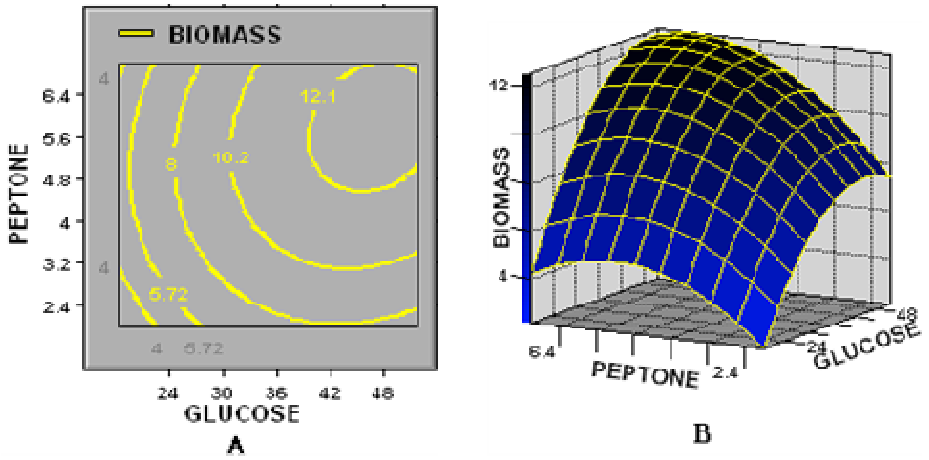

Figure 1. The contour (A) and surface (B) plots of the combined effects of glucose and peptone on the biomass production by *G. sinense*. Fixed level: KH_2PO_4 = 0.

by mainly considering the significant terms and was expressed by Equation (2):

$$Y_{biomass}=10.99+1.96x_1+0.96x_2+0.23\ x_3-0.95\ x_1^2+0.3x_1x_2-0.75x_2^2-0.95x_3^2 \quad (2)$$

Table 4 and equation (2) reveal that glucose concentration (x_1) had a strong positive linear effect on the response ($P < 0.01$) on $Y_{biomass}$ as it had the largest coefficient, followed by peptone (x_2). However, KH_2PO_4 (x_3) had no significant effect on the biomass production at the tested concentrations ($P > 0.05$), and the above three variables also indicated negative quadric effects on the biomass yield ($P < 0.01$). No significant interactions were noted between any two of the three variables ($P > 0.05$).

Figure 1 describes the contour (A) and surface (B) plots of the combined effects of glucose and peptone on the biomass production. It was obvious that the mycelial

Table 5. Results of regression analysis of a predictive polynomial model for optimization of EPS production of *G. sinense*.

Factor	Coefficients estimated	t value	Pr > |t|
Intercept	233.633763	22.21	<.0001**
x_1	74.390042	10.87797	0.0001**
x_2	32.303915	4.723762	0.000626**
x_3	4.9473248	0.723441	0.484509
x_1x_1	-24.43414	-3.67033	0.003687**
x_1x_2	12.575	1.407378	0.186939
x_2x_2	-51.74616	-7.77297	0.0001**
x_2x_3	6.75	0.755451	0.465847
x_3x_3	-38.20506	-5.73891	0.00013**

** Significant at 1 % level.

Table 6. Analysis of variance (ANOVA) for full quadratic model for optimization of EPS production of *G. sinense* R^2 = 0.9589; Adj. R^2 = 0.9188.

Regression	DF	Sum of squares	R-square	F value	Pr > F
Linear	3	94183	0.5818	47.20	<0.0001**
Quadratic	3	59105	0.3651	29.62	<0.0001**
Crossproduct	3	1950.1650	0.0120	0.98	0.4416
Total model	9	155238	0.9589	25.93	<0.0001**

** Significant at 1 % level.

growth of *G. sinense* was sensitive even when glucose and peptone concentration was subject to small alteration. An increase in biomass yield could be significantly achieved with the increases of glucose and KH_2PO_4 concentration. The model predicted the maximum biomass yield of 12.58 g/l appeared at glucose, peptone, KH_2PO_4 of 42.0 5.28 and 0.77 g/l respectively.

Optimization of EPS production

Table 5 shows the results of regression analysis of a full second-order polynomial model for optimization of EPS production. Judging from the regression coefficients and mainly considering the significant terms, we obtain the polynomial model for EPS yield:

$$Y_{EPS} = 233.63 + 74.39x_1 + 32.31x_2 - 24.43\ x_1^2 + 12.58\ x_1x_2 - 51.75x_2^2 - 38.20\ x_3^2 \quad (3)$$

As shown in Table 5, both glucose and peptone concentration (x_1 and x_2) had significant positive linear effects ($P < 0.001$), and as indicated in equation (3), glucose concentration had the largest coefficient, followed by peptone (x_2). Positive coefficient of x_1 and x_2 (x_1x_2) indicated a linear effect to increase Y_{EPS}. However, quadratic terms (x_1^2, x_2^2 and x_3^2) had negative effects, and x_3 had no significant positive linear effect ($P > 0.05$) on the EPS yield. The goodness of fit model was also examined by determination coefficient (R^2 = 0. 9589), which implied that the sample variation of more than 95% was attributed to the variables (Table 6). The test statistics F values for the overall regression is significant at the upper 1% level, which further supported that the second-order model was very adequate in approximating the response surface of the experimental design (Table 6). As an example, the contour (A) and surface (B) plots of the combined effects of glucose and peptone on the EPS production was shown in Figure 2. Canonical analysis revealed a maximum of EPS yield of 309.6 mg/l appeared at glucose, peptone, KH_2PO_4 of 45.4, 4.98 and 0.79 g/l respectively.

Verification of the models

Verification of the calculated optimum model (Equation 2) for biomass production was done with a culture medium representing this maximum point and yielding biomass 12.36 g/l (average of three repeats). The excellent correlation between predicted and measured values of these experiments justifies the validity of the response model and the existence of an optimum point. The triplicate experiments were also carried out to verify the

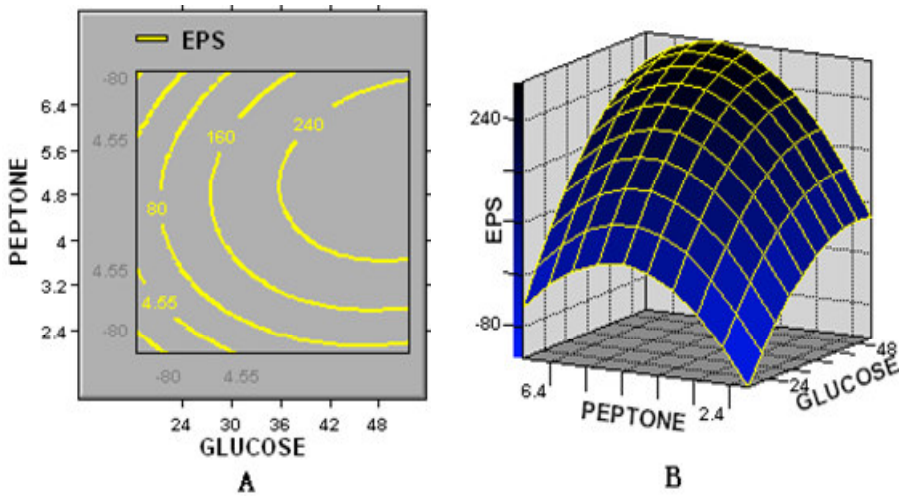

Figure 2. The contour (A) and surface (B) plots of the combined effects of glucose and peptone on the EPS production by *G. sinense*. Fixed level: KH_2PO_4 = 0.

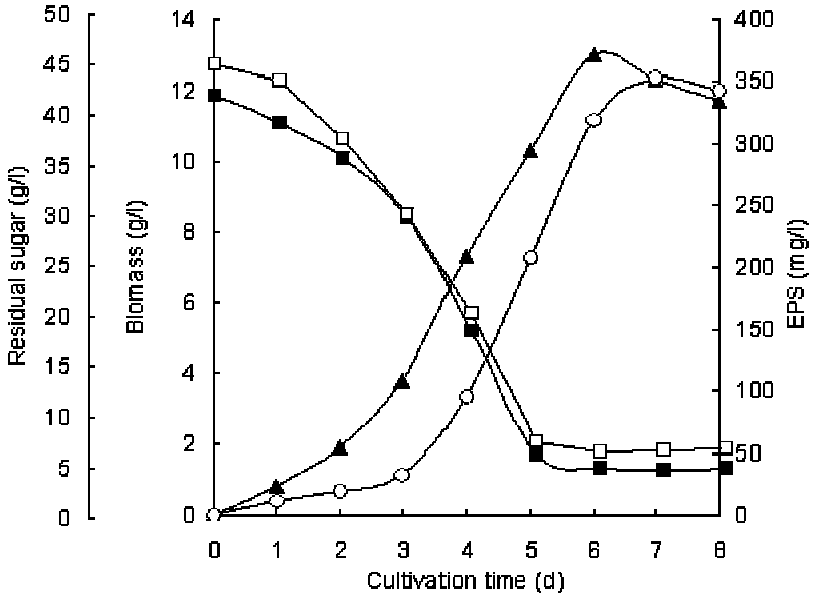

Figure 3. Time course of mycelial growth (▲) at medium A, polysaccharide production (○) at medium B and sugar consumption under medium A (■) and B (□) by *G. sinense* growth in a 30-l stirred-stank bioreactor. Medium A (g/l): glucose, 42.0; peptone, 5.28; KH_2PO_4, 0.77; $MgSO_4 \cdot 7H_2O$, 0.4, and vitamin B_1, 0.01. Medium B (g/l): glucose, 45.4; peptone, 4.98; KH_2PO_4, 0.79; $MgSO_4 \cdot 7H_2O$, 0.4, and vitamin B_1, 0.01.

availability and accuracy of the model (Equation 3) for EPS production in a similar way as that of the biomass production model. Under the calculated optimal culture composition, EPS production was 315.3 mg/l, which was in agreement with the predicted value (309.6 mg/l), suggesting the model (Equation 3) was also very valid for EPS production.

Bioreactor fermentation results

The feasibility of the two regression models in a 30-l scaled fermenter was also tested under the optimized medium. Figure 3 shows the time course of biomass production using the calculated optimal medium (Medium A), and EPS production under its optimal culture

composition (Medium B). After 5 days fermentation, the concentration of residual sugar in biomass and EPS fermented broth sharply decreased to 6.3 and 7.6 g/l, respectively. The maximum of biomass yield (13.01 ± 0.36 g/l) was obtained at 6th d, while the maximum of EPS yield (352 ± 7.9 mg/l) appeared at 7th d.

DISCUSSION

As *Ganoderma* is very rare in nature, the amount of wild mushroom is not sufficient for commercial exploitation. Its cultivation on solid substrates, stationary liquid medium or, in the last time, by submerged cultivation has become essential to meet the increasing demands in the international markets (Hsieh et al., 2005; Tang et al., 2009). Currently, most of the research has been related to *G. lucidum* (Fang and Zhong, 2002; Liu and Zhang, 2007; Tang et al., 2009; Zhang and Zhong, 2010), however, data on submerged cultivation of *G. sinense* are scarce.

Our previous study indicated glucose, peptone and KH_2PO_4 were significant variables for biomass and EPS production (Wang and Liu, 2009). In this work, the critical medium ingredients of *G. sinense* were optimization using response surface methodology, and the results showed that at optimal medium, the experimental biomass and EPS yields were 12.36 g/l and 315.3 mg/l in flask cultures, respectively, which were 137% and 156% of those obtained in our previous work (Wang and Liu 2009). The excellent correlation between predicted and measured values of each model justifies the validity of both the response models. The results of bioreactor fermentation also showed that the optimized culture medium could enhance both biomass (13.01± 0.36 g/l) and EPS (352 ± 7.9 mg/l) production in a large scaled fermentation process.

In conclusion, the present study indicated RSM is an effective method for maximum production of biomass and EPS and provided useful information for the production of the two products by *G. sinense* on a further large scale.

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