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Full Length Research Paper

Statistical optimization of the key medium components by response surface methodology to promote ganoderic acid formation by medicinal mushroom *Ganoderma sinense* in submerged culture

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Statistical experimental design was used to optimize ganoderic acid (GA) production of the medicinal mushroom, *Ganoderma sinense* in submerged culture. A central composite design (CCD) was employed to determine ganoderic acid (GA) yield at optimum levels for the key medium compositions, sucrose, peptone and yeast extract (YE). A mathematical model was then developed to show the effect of each medium composition and their interactions on the production of GA. The model predicted a maximum of GA yield of 249.2 mg/l appeared at sucrose, peptone, YE of 39.6 g/l, 4.0 g/l, 1.1 g/l, respectively, and a maximum of GA yield of 246.6 mg/l in validation experiment were obtained, which represented a 30.5 % increase in titre compared to that of the non-optimized medium. The amounts of glucose, peptone and YE required were also reduced with the statistical optimization. In addition, 261.7 mg/ of GA was obtained in a 30-l scaled fermenter under the optimized medium, suggesting that the medium optimized in the study was also suitable for GA production in a large scale fermentation process.

Key words: Medicinal mushroom, *Ganoderma sinense*, Submerged culture, response surface methodology, ganoderic acid.

INTRODUCTION

Ganoderma, a genus of medicinal polypore mushroom, is highly regarded in Chinese herbal medicine texts due to their extensive use in traditional Chinese medicines for the prevention and treatment of various types of diseases, such as cancer, hepatopathy, arthritis, hypertension, neurasthenia, and chronic hepatitis (Liu and Zhang, 2005; Lin, 2007). Among the genus, two key species, *G. lucidum* and *G. sinense* are indexed in the Pharmacopoeia of the Peoples Republic of China, and used as key herbal medicine in China for centuries (Lin, 2007). Modern chemistry studies shown that polysaccharides and triterpenoids (especially ganoderic acid, GA) are the major source of biological activity and therapeutic use of *G. lucidum* and *G. sinense* (Lin, 2007; Sato et al., 2009a; Liu et al., 2010; Qiao et al., 2007; Xu et al., 2010).

Recent studies show that the triterpenoids or GAs from *G. sinense* and *G. lucidum* have various biological functions, such as cytotoxicity to several cancer cells *in vitro*, or inhibition of tumor invasion *in vitro* and *in vivo* (Chen et al., 2010; Min et al., 2000; Kimura et al., 2002), inhibition of HIV-1 protease (Sato et al., 2009b), inhibition of eukaryotic DNA polymerases (Mizushina et al., 1999), inhibition of cholesterol synthesis and absorption (Miyamoto et al., 2009, regulation of osteoclastogenesis), and inhibition of U46619-induced platelet aggregation (Chen et al., 1999).

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There is a great need to supply the market with high-quality of *G. sinense* and *G. lucidum* products 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 (Liu et al., 2010). Currently, submerged fermentation of the two fungi is viewed as a promising alternative for the efficient production of mycelial biomass, polysaccharides and ganoderic acid (GA). In recent years, most of the research has been related to *G. lucidum*, and to obtain metabolites from *G. lucidum*, the environmental conditions of fermentation, two-stage culture process, *etc*. have been studied (Yang and Liau, 1998; Fang and Zhong, 2002; Tang et al., 2009; Zhang and Zhong, 2010). However, data on efficient submerged cultivation of *G. sinense* are scarce, and until now, little attention has been paid to the optimization of the critical medium components for GA production by *G. sinense* in submerged fermentation, and the mutual interactions between medium constituents on GA 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; Liu and Wang 2007; Liu et al., 2010). 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, 2005), we evaluated the suitability of various carbon sources and nitrogen sources for the effective production of GA by *G. sinense*. The preliminary data indicated that the major constituents affecting the performance of culture in terms of GA yields were the concentrations of sucrose, peptone and yeast extract (YE) (Wang, 2005). Here, we further report high GA production as a result of interactions among the three variables (sucrose, peptone and YE) using central composite design (CCD) and response surface methodology (RSM).

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.

Shake flask culture

The culture medium was composed (g/l) of: Sucrose 40, peptone 4, YE 1, KH_2PO_4 0.5, $MgSO_4 \cdot 7H_2O$ 0.3, 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°C in a rotatory shaker at 160 rpm for 8 days to prepare the inoculums. A 10% (v/v) inoculum was added to the flask (500 ml) containing 100 ml of medium. The fermentation was incubated at 160 rpm at 30°C for 7 days.

Bioreactor culture

The bioreactor culture was carried out in a mechanical agitated 30-L fermenter (made in Jiangsu University, China), under the following conditions: Medium volume 20 L, inoculation volume 10% (v/v), temperature 30°C, aeration rate 8.0 vvm, and agitation speed 180 rpm. The culture continued until the dry cell weight and GA reached their highest values and the residual sugar concentration did not further decrease.

Determination of dry cell weight

Samples collected from flasks were filtered using a 40-mesh stainless sieve and the mycelium was harvested. Mycelial biomass was collected by centrifuging the mycelium at 8000 rpm for 15 min, washing the precipitated cells for three times with distilled water, and drying at 60°C until it reached a constant weight (Liu and Wang, 2007).

Measurement of GA from mycelium

The determination of GA was made by a method described by our previous work (Liu et al., 2010). The dried mycelia (2 g) were extracted by circumfluence with 50% (v/v) ethanol (100 mL) for 2 h (twice). After removal of mycelia by centrifugation, the supernatant was dried at 50°C under vacuum. The residues were suspended in water, and later extracted with chloroform. The GA in chloroform was extracted by 5% (w/v) $NaHCO_3$. After adding 2M HCl to adjust the pH of the $NaHCO_3$ layer to be lower than 3, the GA in the $NaHCO_3$ layer was extracted with chloroform. After removal of chloroform by evaporation at 40°C, GA was dissolved in absolute ethanol, and its absorbance was measured at 245 nm.

Measurement of residual sugar

After removal of mycelia by centrifugation, the supernatant was dried at 50°C under vacuum. The residues were hydrolized by HCl and 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 that sucrose, peptone and YE were significant variables for GA production. The concentration of KH_2PO_4, $MgSO_4 \cdot 7H_2O$ and V_{B1} were kept constant throughout the investigation since they had no significant effect on GA production (Wang, 2005).

A central composite design (CCD) was used in the optimization of GA production. The range and levels of the variables investigated in this study are given in Table 1. The lowest and the highest levels of variables were: Sucrose, 24.89 and 50.11 g/l; peptone 2.32 and 5.68 g/l; YE, 0.43 and 1.77 g/l.

A mathematical model, describing the relationships between the process indices (the yield of mycelial biomass and GA) and the medium component contents in second-order equation, was developed. The yield of mycelial biomass and GA by *G. sinense* was multiply regressed with respect to the fermentation

Table 1. Experimental range and levels of the independent variables of ganoderic acid (GA) optimization.

Independent variables (g/l)	Range and levels				
	-1.682	**-1**	**0**	**1**	**1.682**
Sucrose, X_1	24.89	30.00	37.50	45.00	50.11
Peptone, X_2	2.32	3.00	4.00	5.00	5.68
YE, X_3	0.43	0.70	1.10	1.50	1.77

Table 2. The central composite design matrix and the responses of ganoderic acid (GA) of *G. sinense*.

Runs	x_1	x_2	x_3	Y_{GA} (mg/l)
1	-1	-1	-1	36.2
2	-1	-1	1	47.1
3	-1	1	-1	68.3
4	-1	1	1	60.3
5	1	-1	-1	174.2
6	1	-1	1	170.9
7	1	1	-1	181.7
8	1	1	1	177.3
9	-1.682	0	0	59.3
10	1.682	0	0	158.2
11	0	-1.682	0	136.9
12	0	1.682	0	159.1
13	0	0	-1.682	171.3
14	0	0	1.682	181.3
15	0	0	0	239.3
16	0	0	0	241.3
17	0	0	0	234.1
18	0	0	0	231.3
19	0	0	0	240.7
20	0	0	0	233.6

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, $i \neq j$) 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. Tables 2 showed the central composite design matrix and the responses of biomass and GA.

SAS statistical package (version 8.1, USA) was performed for regression and graphical analysis of data obtained. The optimum concentrations of sucrose, peptone and YE were obtained by solving the regression equation.

RESULTS

The effects of three variables on GA production of *G. sinense* were studied. So far, this was the first time the medium constituents had been optimized for GA production using RSM. The experimental design matrix is presented in Tables 1 and 2. Twenty experiments were performed in triplicate. Table 3 shows the analysis of variance for the experiment. The Fisher's F-test with a very low probability value [($P_{model} > F$) = 0.0001] for total model indicated that the model was highly significant, and the coefficient of determination (R^2) was shown as 0.9419, indicating that 94.19 % of the variability in the response could be explained by the model. The adjusted determination coefficient (Adjusted R^2 = 0.8896) was also satisfactory to confirm the significance of the model (Tables 1, 2 and 3).The significance of each coefficient was determined by Student's *t*-test and *P*-value, which is listed in Table 4. The larger the magnitude of *t*-test and smaller the *P*-value, the more significant is the corresponding coefficient. The polynomial model for GA yield Y_{GA} was regressed by mainly considering the significant terms and was expressed by Equation (2). The regression equation showed that the GA yield was an

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

Regression	DF	Sum of squares	*R*-Square	*F* value	*Pr* > *F*
Linear	3	32447	0.3445	19.77	0.0002**
Quadratic	3	56069	0.5954	34.17	<.0001**
Crossproduct	3	187.2900	0.0020	0.11	0.9498
Total model	9	88704	0.9419	18.02	0.0001**

R^2 = 0.9419, Ajusted R^2 = 0.8896; **Significant at 0.01 level.

Table 4. Results of regression analysis of a predictive polynomial model for optimization of ganoderic acid (GA) of *G. sinense.*

Parameter	DF	Coefficients estimated	Standard error	t value	Pr > |t|
Intercept	1	237.499604	9.538928	24.90	<0.0001**
x_1	1	48.219739	6.328866	7.62	<0.0001**
x_2	1	7.068676	6.328866	1.12	0.2902
x_3	1	0.879992	6.328866	0.14	0.8922
x_1x_1	1	-50.359865	6.160992	-8.17	<0.0001**
x_1x_2	1	-3.925000	8.269057	-0.47	0.6452
x_2x_2	1	-36.482847	6.160992	-5.92	0.0001**
x_2x_3	1	-2.500000	8.269057	-0.30	0.7686
x_3x_3	1	-26.477253	6.160992	-4.30	0.0016**

** Significant at 0.01 level.

empirical function of test variables in coded unit.

YGA = 237.50 + 48.22 x1 + 7.07 x2 + 0.88 x3 - 50.36 x12 - 3.93 x1x2 - 36.48 x22 - 2.5 x2 x3 - 26.48 x32 (2)

where Y_{GA} is the predicted GA yield, x_1 sucrose, x_2 peptone, and x_3 is YE.

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

The 2D contour plot and 3D response surface are generally the graphical representation of the regression equation. Figures 1 to 3 represent the 2D contour plots (A) and 3D response surfaces (B) for the optimization of medium components of GA production. Each figure presented the effect of two variables on the production of GA, while other two variables were held at zero level.

The model predicted the maximum GA yield of 249.2 mg/l appeared at sucrose, peptone and YE of 39.6, 4.0 and 1.1 g/l, respectively.

Verification of the models

The triplicate experiments were carried out to verify the availability and accuracy of the model (Equation 2) for GA production. Under the calculated optimal culture composition, GA production was 246.6 mg/l, which represented a 30.5% increase in titre compared to the non-optimized medium (Wang, 2005), and was also in agreement with the predicted value (249.2 mg/l), suggesting that the model (Equation 2) was very valid for GA production.

Bioreactor fermentation results

The feasibility of the regression model was further tested in a 30-L scaled fermenter under the optimized medium. The time courses of mycelial growth, GA production and substrate consumption were recorded as Figure 4. It is shown that the concentrations of residual sugar in the 30-l scaled bioreactor sharply decreased to 7.1 g/l on the 5th day and 4.5 g/l in the 6th day. The maximum of GA yield (261 ± 3.7 mg/l) appeared on the 7th day, suggesting that the medium optimized in the study was also suitable for GA production in a large scale. In addition, a maximum of dry cell weight (11.21 ± 0.63 g/l) was obtained on the 6t^{h} day (Figure 4).

DISCUSSION

Mushrooms have recently become attractive as healthy foods (physiologically functional) and as a source material for the development of drugs. *G. sinense*, a

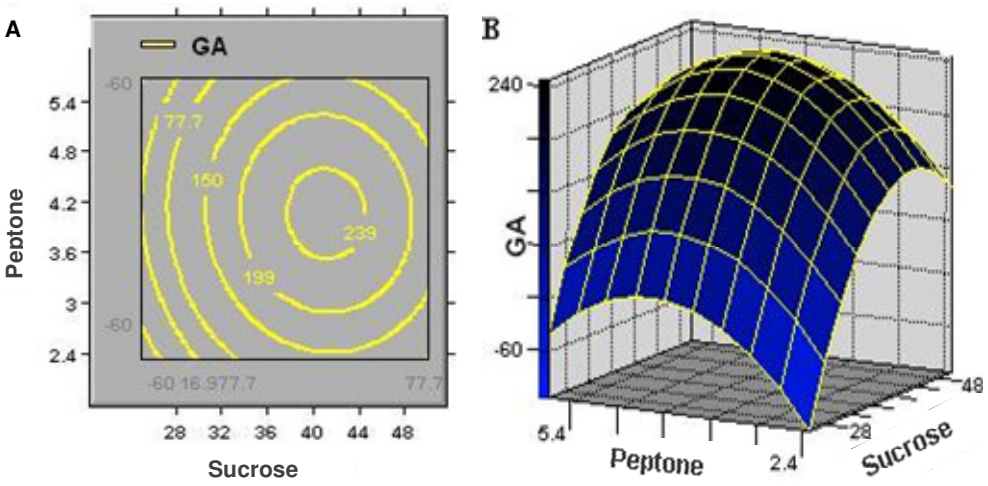

Figure 1. The contour (A) and surface (B) plots of the combined effects of sucrose and peptone on GA production by *G. sinense*. Fixed level: YE = 0.

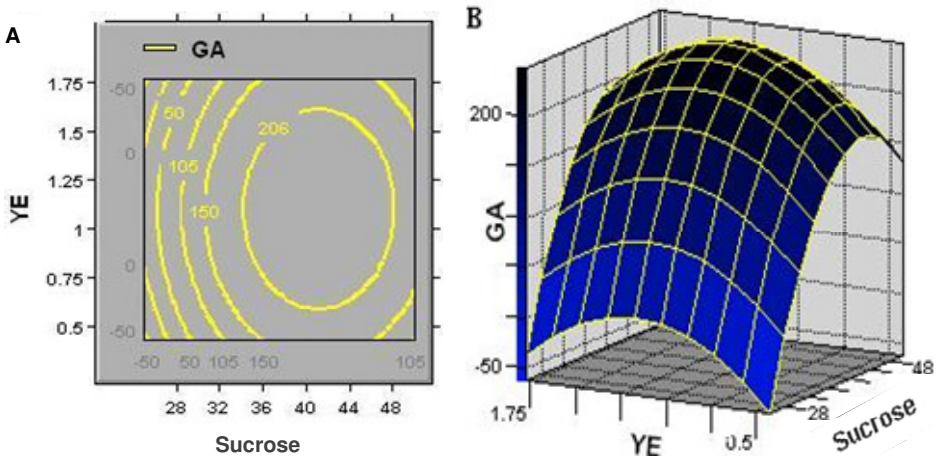

Figure 2. The contour (A) and surface (B) plots of the combined effects of sucrose and YE on GA production by *G. sinense*. Fixed level: peptone = 4.0.

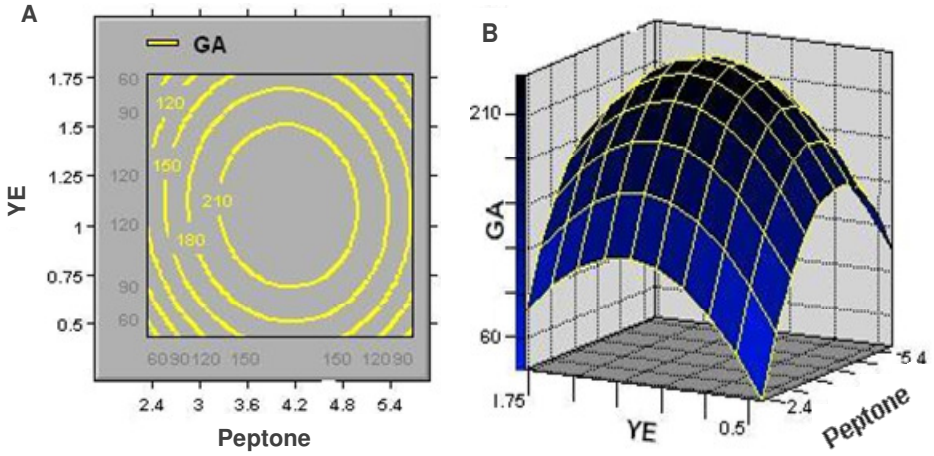

Figure 3. The contour (A) and surface (B) plots of the combined effects of peptone and YE on GA production by *G. sinense*. Fixed level: sucrose = 37.5.

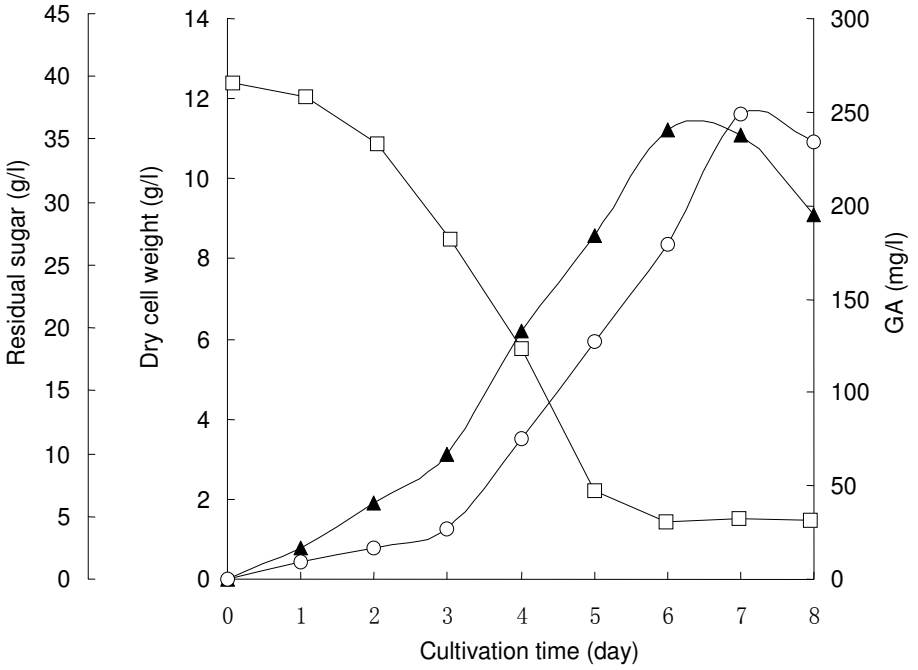

Figure 4. Time course of sugar consumption(□), mycelial growth (▲) and ganoderic acid (GA) production (○) under the optimized medium by *G. sinense* growth in a 30-l stirred-stank bioreactor. Temperature 30℃, aeration rate 8.0 vvm, and agitation speed 180 rpm.

medicinal mushroom, is indexed in the Pharmacopoeia of the Peoples Republic of China (PPRC), and used as key herbal medicine in China for centuries (Lin, 2007). In the last decade, the isolation, chemical structures and pharmacological actions of pharmaceutically active compounds from *G. sinense* were studied. However, submerged cultivation of *G. sinense* had not been well developed, and studies have been focused on the polysaccharide production (Wang and Liu, 2009), little attention was payed to the production of another key active compound, GA of *G. sinense* in submerged fermentation.

In a previous study, we screened the stimulators from Chinese medicinal insects for mycelial growth and ganoderic acid (GA) production by *G. sinense*, and found that the ether extract of medicinal insect *Eupolyphaga sinensis* at a concentration of 60 $mg \cdot L^{-1}$ lead to a significant increase in GA concentration (Liu et al., 2010). Our previous study indicated that sucrose, peptone and YE were significant variables for GA production (Wang, 2005). In the present work, the critical medium variables for GA production by *G. sinense* and the mutual interactions between the variables on GA production are studied. The results showed that at optimal medium, the experimental GA yield was significantly enhanced compared with that of the non-optimized medium (the production of GA was enhanced 30.5%, experimentally), and the excellent correlation between predicted and measured values of the mathematical model justifies the validity of the response model, suggesting that the chosen method of optimization of medium composition was efficient. Moreover, in the optimized complex medium, the amounts of glucose, peptone and YE required were reduced to 5.4, 0.5 and 0.4 g/l, respectively, which would in turn reduce the cost of the medium. In addition, the validity of the response model was justified in a 30-L scaled fermenter, suggesting that the medium optimized in the study was also suitable for GA production in a large scale fermentation process.

In conclusion, so far, there are no reports of GA production from *G. sinense* by media engineering. The results indicated that RSM is an effective method for maximum production of GA. The optimization of the medium resulted not only in a 30.5% higher GA concentration than non-optimized medium, but also in success in a large scale fermentation process, and in a reduced amount of the medium constituents. The chosen method of optimization of medium composition was efficient, relatively simple and material saving.

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