Statistical Optimization of the Medium Composition by Response Surface Methodology to Enhance Schizophyllan Production by *Schizophyllum commune*

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The response surface methodology (RSM) involving central composite design (CCD) was employed to optimize the fermentation medium for the cell growth and schizophllan production by *Schizophyllum commune* CGMCC 5.113 in submerged culture at pH 6.5 and 26 °C. The four variables involved in this study were glucose, yeast extract, ammonium nitrate, and magnesium sulfate. The statistical analysis of the results showed that, in the range studied, glucose and yeast extract had a highly significant effect on schizophyllan production. The optimal medium for schizophyllan production calculated from the regression model of RSM was as follows: glucose, 18 g/l; yeast extract, 0.5 g/l; NH_4NO_3, 0.48 g/l; and $MgSO_4$, 0.05 g/l, with a predicted maximum schizophyllan production of 11.74 g/l. These predicted values were experimentally validated. The excellent correlation between predicted and measured values justifies the validity of the response model. The results of bioreactor fermentation also show that the optimized medium enhanced schizophyllan production (12.80 g/l) by *S. commune* in a 5-l fermenter.

Key words: Schizophyllan, Response Surface Methodology, Medium Optimization

Introduction

Schizophyllum commune is a species of the basidiomycetes which belongs to the order Agaricales and the family Schizophyllaceae (Bolla *et al.*, 2008; Hao *et al.*, 2010). It is a very common fungus and has a world-wide distribution (Hobbs, 1995). In particular, this fungus has been regarded as a popular healthy food and an effective medicine used in the therapy of some diseases in the Orient for centuries. Pharmacologically it is extremely important because it produces the polysaccharide schizophyllan (Rau, 1999), a homoglucan consisting of a linear chain of β-D-(1→3)-glucopyranosyl groups and β-D-(1→6)-glucopyranosyl groups, produced by fermentation of filamentous *S. commune* (Rau, 2002). This polysaccharide has attracted much attention by pharmaceutical industry in recent years because its immunomodulatory, antineoplastic and antiviral activities are higher than those of other glucans (Kumari *et al.*, 2008; Tabata *et al.*, 1981). Furthermore, schizophyllan has been applied in enhanced mineral oil recovery (Leathers *et al.*, 2006; Wagner, 1988), in cosmetics (Rau and Brandt, 1994), and food preservation (Hao *et al.*, 2010; Leathers *et al.*, 2006). Currently, schizophyllan is commercially produced in Japan as an antitumour agent with the trade name Sizofiran (Leathers *et al.*, 2006). Although its structure and its applications have been well documented in the literature in the last decade, relatively little research has focused on factors affecting the production of schizophyllan. To achieve higher yields in submerged culture, it is a prerequisite to design an optimal production medium.

The optimization of a fermentation medium is an important step in the development of economically feasible bioprocesses. The successful design of a fermentation process involves optimizing the media composition, fermentation conditions, and fermenter design as well as developing superior strains by mutation (Margaritis and Pace, 1985). Medium optimization by employing the one-fac-

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tor-at-a-time method involves changing one independent variable while fixing all the others at a certain level. This single-dimensional approach is laborious and time-consuming, especially for a large number of variables, and frequently does not guarantee the determination of optimal conditions (Luo *et al.*, 2009; Survase *et al.*, 2006). Such drawback of the one-factor-at-a-time method can be overcome by statistical optimization techniques (Chen *et al.*, 2008). Factorial design and response surface methodology (RSM) are important statistical optimization methods for the optimization of many variables by only a few experimental trials (Malinowska *et al.*, 2009).

RSM is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors, and searching for the optimum conditions. RSM has been successfully used in the optimization of bioprocesses where the target production could be enhanced, especially in the cultivation process of many edible and medicinal mushrooms (Hao *et al.*, 2010; Kalil *et al.*, 2000; Kumari *et al.*, 2008; Luo *et al.*, 2009). However, there is still lack of knowledge concerning the optimization of the medium for schizophyllan production by *Schizophyllum commune* in submerged culture by statistical techniques.

The aim of the present study was to establish the optimal medium for the production of schizophyllan by *Schizophyllum commune* using RSM. In the first step, the one-factor-at-a-time method was used to investigate the effect of media components. Subsequently, an optimal medium composition was attained by full 2^4 factorial plus 7 centre points central composite design (CCD) using RSM. Finally, with the optimal medium, the cell growth (biomass) and schizophyllan production were investigated in a 5-l fermenter.

Material and Methods

Microorganism

Schizophyllum commune (CGMCC 5.113) used in this study was kindly provided by the China General Microbiological Culture Collection of the Chinese Academy of Sciences. The stock culture was maintained on a potato dextrose agar (PDA) slant containing (per liter): 200 g potato juice, 20 g glucose, 0.5 g $MgSO_4 \cdot 7\ H_2O$, 3.0 g KH_2PO_4, and 20 g agar, and subcultured once a month. The slants were incubated at 25 °C for 7 d, and then stored at 4 °C.

Flask culture

The culture medium selected for studies on schizophyllan production comprised the following (per liter): 12 g glucose, 1 g yeast extract, 0.2 g KH_2PO_4, 0.1 g $MgSO_4 \cdot 7\ H_2O$, pH 6.5. The pH value of the medium was initially adjusted to 6.5, followed by its autoclaving at 121 °C for 20 min. The seed culture obtained by transferring approximately 1.0 cm^2 of slant culture into a 250-ml Erlenmeyer flask containing 100 ml seed medium was grown at 28 °C on a rotary shaker at 150 rpm for 5 d.

The flask culture experiments were performed in 250-ml flasks, containing 100 ml of the above medium. After inoculating with 10% (v/v) of seed culture, the culture was incubated at 26 °C in a rotary shaker incubator at 180 rpm for 7 d.

Bioreactor fermentation

The bioreactor fermentation was carried out in a 5-l fermenter (BIOSTAT B plus, B. Braun Biotech International, Melsungen, Germany) with a working volume of 3 l and 10% inoculation volume (v/v) at 26 °C for 7 d. The composition of the medium was optimal for schizophyllan production calculated from the result of RSM. The agitation rate remained at 180 rpm. The culture was aerated at a rate of 1 vvm and the pH value was controlled using 1 M NaOH and 1 M HCl.

Optimization of fermentation medium using the one-factor-at-a-time method

The effects of carbon source, nitrogen sources, and the initial pH value on schizophyllan production were examined by the one-factor-at-a-time method. Glucose was substituted with six different carbon sources *viz*., sucrose, maltose, fructose, lactose, soluble starch, and corn starch. Initially, all carbon sources were screened at 12 g/l. Yeast extract was replaced with other organic nitrogen sources such as peptone, acid-hydrolyzed casein, and inorganic nitrogen sources like sodium nitrate, ammonium nitrate, and urea at the nitrogen concentration of 1 g/l. The medium was adjusted to different pH levels varying from 5.0 to 8.0 with 1 M NaOH and 1 M HCl. Fermentation was carried out at 26 °C and 180 rpm for 7 d.

Optimization of medium components by RSM

RSM is a statistical modeling technique used for multiple regression analysis of quantitative

data obtained from rationally designed experiments to solve multivariable equations simultaneously (Kumari *et al.*, 2008; Rao *et al.*, 2000). The ingredients concentrations of medium for the production of schizophyllan were optimized by RSM. The experiments were designed with CCD using the software Design Expert Version 7.0.0 trial version (State Ease, Minneapolis, MN, USA). The culture medium components (independent variables) selected for optimization were carbon source, nitrogen source, and mineral elements. Regression analysis was performed on the data obtained from the design experiments. The following equation was used to code the test variables:

$$X_i = \frac{x_i - x_i^0}{\Delta x_i},\ i = 1, 2, 3, \cdots, k, \qquad (1)$$

where X_i is the dimensionless coded value of the i-th test variable, x_i is the real value of the i-th test variable, x_i^0 is the real value of the i-th test variable at the centre point, and Δx_i is the step change value.

The relationship between the independent variables (nutrient medium components) and the response (schizophyllan production) was fitted to a predictive second-order polynomial equation:

$$Y = \beta_0 + \sum_{i=1}^{k}\beta_i X_i + \sum_{i=1}^{k}\beta_{ii}X_i^2 + \sum_{i<1}^{k}\sum\beta_{ij}X_iX_j, \qquad (2)$$

where Y is the predicted response, subscripts i and j assume values from 1 to the number of variables, β_0 is a constant, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, β_{ij} is the cross-product coefficient, k is the number of factors, and X_i and X_j are the coded dimensionless values of the investigated variables. The software mentioned above was used for the experimental design, the analysis of variance (ANOVA), and the graphical analysis of the data. The statistical significance of the quadratic model was assessed using the F-test, and the quality of fit was evaluated by the coefficient of determination R^2. The significances of the regression coefficients were tested by a t-test, and the p values were used as a tool to check the significance of each coefficient.

Determination of fungal biomass and schizophyllan production

A flask containing 50 ml fermentation broth was taken from the shaker. Broth (10 ml) from the flask was filtered to separate fungal biomass, which was washed three times with distilled water and dried at 65 °C to constant weight; it was reported as dry cell weight (DCW). The supernatant was used for estimation of schizophyllan dry weight (Hsieh *et al.*, 2005). Two volumes of absolute ethanol were added to precipitate the polysaccharide from the clear supernatant. The mixture was allowed to stand for 12 h at 4 °C for complete precipitation. The precipitated polysaccharide was collected by centrifugation at 6,000 × g for 15 min, then dried at 65 °C to remove the residual ethanol, and quantified as dry weight (Maziero *et al.*, 1999).

Results and Discussion

Effect of carbon

During microbial culture, the carbon source functions as a source of both energy and constituent cellular material (Kumari *et al.*, 2008). Fig. 1 shows the effect of different carbon sources on schizophyllan and biomass production by *S. commune*. Glucose and sucrose supported the mycelial growth best. Glucose allowed the highest production of 11.20 g/l schizophyllan, whereas sucrose gave a yield of 10.20 g/l schizophyllan after 7 d of cultivation. Likewise, glucose provided maximal biomass (measured as DCW), while corn starch was least effective. Glucose has also been identified as being ideally suited for exopolysaccharide (EPS) production in fungi such as *Agaricus brasiliensis* (Shu and Xu, 2007), *Grifola fron-*

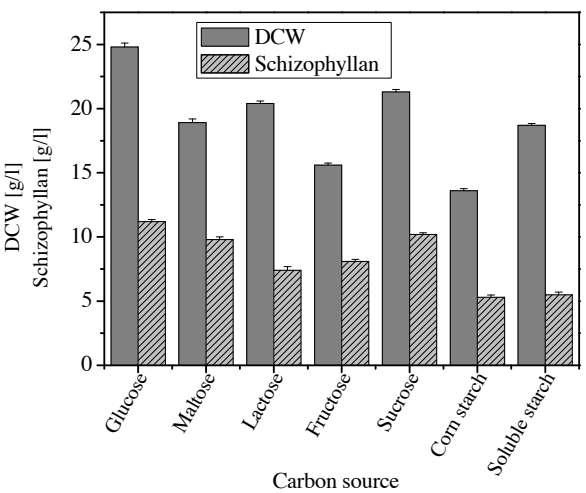

Fig. 1. Effect of different carbon sources on schizophyllan and biomass production (DCW) by *S. commune*. (Carbon source, 12 g/l; yeast extract, 1 g/l.)

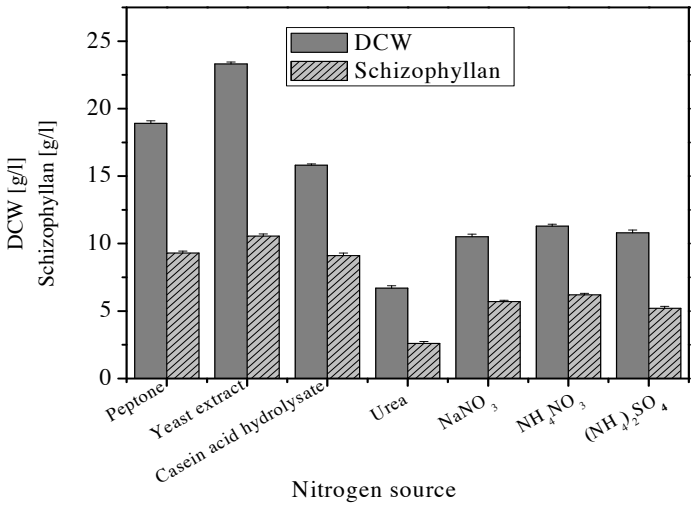

Fig. 2. Effect of different nitrogen sources on schizophyllan and biomass production (DCW) by *S. commune*. (Nitrogen concentration, 1 g/l; glucose, 12 g/l.)

dosa (Lee *et al*., 2004), and *Pleurotus pulmonarius* (Nour El-Dein *et al*., 2004).

Effect of the nitrogen source

Fig. 2 shows the effect of different organic and inorganic nitrogen sources (1 g/l, calculated as nitrogen) on schizophyllan and biomass production by *S. commune*. Nitrogen is a critical factor in the synthesis of some fungal enzymes involved in both primary and secondary metabolism (Malinowska *et al.*, 2009). Limiting nitrogen in the medium can result in considerable inhibition of cell growth and metabolite production (Kim *et al*., 2005). This nutrient element can be supplied to the culture medium in the form of ammonium or nitrate ions, or in organic form (such as free amino acids or proteins).

Among the three selected organic nitrogen sources (yeast extract, peptone, and acid-hydrolyzed casein), yeast extract gave the highest yield of 10.56 g/l schizophyllan. Among the four inorganic nitrogen sources, ammonium nitrate provided the highest yield of 6.2 g/l schizophyllan. Considerably lower fungal growth was observed in culture medium containing urea as nitrogen source. Ammonium nitrate, an inexpensive nitrogen source, gave better results and was used in the subsequent experiments.

Effect of the initial pH

The effect of different initial pH values on schizophyllan and biomass production was studied. As shown in Table I, an initial pH of 6.5 supported the highest production of 10.84 g/l schizophyllan, whereas maximum biomass production was obtained at pH 7.0. Kumari *et al*. (2008) observed a similar relationship with *S. commune* NRCM. The one-way ANOVA for different initial pH values experiments shows that initial pH values exhibit an excellent correlation with biomass and schizophyllan productions, with high F values (31.93 and 6.20 for biomass and schizophyllan production, respectively) and $p < 0.05$. The initial pH value of the medium has significant effects on biomass growth and schizophyllan production. As different authors have reported different optimal pH values for EPS production, it seems that changes in this environmental factor lead to certain changes in the EPS yield that

Table I. Effect of initial pH value on biomass and schizophyllan production in flask culture of *S. commune*.

pH	Biomass [g/l]	Schizophyllan [g/l]
5.0	18.96 ± 0.82	9.03 ± 1.4
5.5	20.08 ± 0.42	8.74 ± 0.9
6.0	21.38 ± 0.53	9.86 ± 1.6
6.5	21.48 ± 0.36	10.84 ± 0.68
7.0	21.89 ± 0.9	8.76 ± 1.1
7.5	19.82 ± 0.8	6.64 ± 1.0
8.0	15.32 ± 0.78	5.86 ± 1.5

All experimental data shown here are the mean ± SD of triplicate determinations. Means are significantly different according to one-way ANOVA at $p < 0.05$.

Table II. The coded and real values of variables of medium composition.

Independent	Variable levels				
	-2	-1	0	1	2
X_1, glucose (g/l)	10.0	12.0	14.0	16.0	18.0
X_2, yeast extract (g/l)	0.5	0.6	0.7	0.8	0.9
X_3, NH_4NO_3 (g/l)	0.1	0.20	0.30	0.40	0.5
X_4, $MgSO_4$ (g/l)	0.05	0.10	0.15	0.20	0.25

vary depending on the mushroom species, which indicates that there are differences in the environmental requirements of various mushroom strains (Cho *et al*., 2006; Huang *et al*., 2007; Kim *et al*., 2005; Malinowska *et al.*, 2009).

Optimizing the medium composition by RSM

The experiments performed by RSM are based on mathematical techniques that allow us to investigate the relationships between variables of medium components. This method has been successfully applied in the optimization of medium compositions and fermentation processes. Based on the results obtained from the one-factor-at-a-time experiments, four factors exerted the greatest effects on cell growth and schizophyllan production. Four medium components at four different levels were selected for optimization. These were: carbon source, two nitrogen sources, and mineral elements (such as $MgSO_4$). The pH value of the culture medium was 6.5, which was

Table III. Central composite rotatable design (CCD) matrix of independent variables and the corresponding experimental results (the response).

Treatment no.	Medium components [g/l]				Schizophyllan [g/l]	
	Glucose	Yeast extract	NH_4NO_3	$MgSO_4$	Predicted	Experimental
1	-1	-1	-1	-1	6.25	5.20
2	1	-1	-1	-1	11.69	12.00
3	-1	1	-1	-1	5.48	4.80
4	1	1	-1	-1	6.57	6.87
5	-1	-1	1	-1	5.75	5.00
6	1	-1	1	-1	12.25	12.40
7	-1	1	1	-1	6.13	5.20
8	1	1	1	-1	8.29	8.80
9	-1	-1	-1	1	7.42	6.40
10	1	-1	-1	1	11.52	11.95
11	-1	1	-1	1	5.61	4.96
12	1	1	-1	1	5.37	5.60
13	-1	-1	1	1	6.47	5.68
14	1	-1	1	1	11.64	11.80
15	-1	1	1	1	5.82	5.00
16	1	1	1	1	6.65	7.20
17	-2	0	0	0	1.76	4.60
18	2	0	0	0	8.03	6.20
19	0	-2	0	0	9.83	10.60
20	0	2	0	0	4.06	4.30
21	0	0	-2	0	10.10	10.66
22	0	0	2	0	10.88	11.33
23	0	0	0	-2	8.63	9.20
24	0	0	0	2	8.15	8.60
25	0	0	0	0	11.54	11.33
26	0	0	0	0	11.54	11.88
27	0	0	0	0	11.54	11.70
28	0	0	0	0	11.54	11.20
29	0	0	0	0	11.54	11.50
30	0	0	0	0	11.54	11.60
31	0	0	0	0	11.54	11.60

Table IV. Analysis of variance (ANOVA) for the experimental results of the central composite design (quadratic model).

Source[a]	Sum of squares	DF[b]	Mean square	*F* Value	*p* Value[c]
Model	248.63	14	17.76	14.10	<0.0001
X_1	58.84	1	58.84	46.71	<0.0001
X_2	49.88	1	49.88	39.59	<0.0001
X_3	0.90	1	0.90	0.71	0.4112
X_4	0.35	1	0.35	0.27	0.6076
X_1X_2	18.84	1	18.84	14.95	0.0014
X_1X_3	1.13	1	1.13	0.90	0.3568
X_1X_4	1.80	1	1.80	1.43	0.2499
X_2X_3	1.35	1	1.35	1.07	0.3167
X_2X_4	1.07	1	1.07	0.85	0.3702
X_3X_4	0.19	1	0.19	0.15	0.7002
X_1^2	79.02	1	79.02	62.72	<0.0001
X_2^2	37.81	1	37.81	30.01	<0.0001
X_3^2	1.99	1	1.99	1.58	0.2272
X_4^2	17.73	1	17.73	14.07	0.0017
Residual	20.16	16	1.26		
Lack of fit	19.85	10	1.98	38.47	0.0001
Pure error	0.31	6	0.052		
Correlation total	268.78	30			

[a] X_1, glucose; X_2, yeast extract; X_3, NH_4NO_3; X_4, $MgSO_4$.
[b] Degree of freedom.
[c] $p < 0.05$ are significant; R^2(predicted) = 0.9250; R^2(adjusted) = 0.8594.

selected as the optimal pH value on the basis of the one-factor-at-a-time experiment. The concentration of KH_2PO_4 was fixed at 0.3 g/l.

Table II depicts the medium components selected and their concentrations. In order to investigate the combined effect of four different medium components (independent variables) on biomass and schizophyllan production, the full-factorial CCD of $2^4 = 16$ plus 7 centre points and $2{\cdot}4 = 8$ star points leading to a total of 31 experiments was used for optimization of the components of the culture medium in submerged cultivation of *S. commune*.

Table III shows the CCD-predicted responses for schizophyllan production and the experimental results. The experimental values obtained from the CCD were regressed by a quadratic polynomial equation. Equation (3) represents the mathematical model relating the production of schizophyllan (yield) with independent process variables, X_i, and the second-order polynomial coefficient for each term of the equation determined through multiple regression analysis using the software Design Expert. The experimental and predicted values of yields of schizophyllan are also given in Table III. The coded values of independent variables are given in Table II.

The results were analysed using ANOVA (Table IV). ANOVA of the quadratic regression model and model *F* value indicates the model to be significant. The model *F* value is calculated as ratio of mean square regression and mean square residual. The model *p* value (Prob > *F*) is very low (0.0001). This again signifies that the model is significant.

The *p* value was used as a tool to check the significance of each of the coefficients, which are necessary to understand the pattern of mutual interactions between the test variables. The *t* ratio and the corresponding *p* values, along with the coefficient estimate, are given in Table IV. Trivial *p* values (less than 0.05) indicate that the model parameters are significant. The coefficient estimates and the corresponding *p* values suggest that, among the test variables used in the study, X_1 (glucose), X_2 (yeast extract), X_1X_2 (glucose·yeast extract), X_1^2 (glucose), X_2^2 (yeast extract), and X_4^2 ($MgSO_4$) are significant model parameters with *p* values less than 0.05. Other parameters are insignificant. The coefficients of independent variables determined for the second-order polynomial model for the schizophyllan production are given as:

$$\text{yield (g/l)} = 11.54429 + 1.56583\,X_1 - 1.44167\,X_2 + 0.19333\,X_3 - 0.12000\,X_4 - 1.08500\,X_1X_2 + 0.26625\,X_1X_3 - 0.33500\,X_1X_4 + 0.29000\,X_2X_3 - 0.25875\,X_2X_4 - 0.11000\,X_3X_4 - 1.66232\,X_1^2 - 1.14982\,X_2^2 - 0.26357\,X_3^2 - 0.78732\,X_4^2. \quad (3)$$

The fit of the model was also expressed by the coefficient of determination, R^2, which was found to be 0.9250, indicating that 92.50% of the variability in the response could be explained by the model, suggesting that the predicted values exhibit a good correlation with experimental data and that the model is suitable and practicable.

Figs. 3a–3c present the three-dimensional response surfaces, which are the graphical representations of equation (3). Each plot shows the effect of two independent variables varying within the experimental range of schizophyllan production, while the other two variables were fixed at their respective centre point levels. Graphs are given here to highlight the roles played by significant factors. From the central point of the contour plot or from the bump of the 3D plot the optimal composition of medium components was identified. The optimal concentrations for the four components as obtained from the maximum point of the model were calculated to be 18 g/l glucose, 0.5 g/l yeast extract, 0.48 g/l NH_4NO_3, and 0.05 g/l $MgSO_4$, respectively. The predicted maximum yield of schizophyllan was 11.74 g/l under the optimum condition, which is in close agreement with the experimental value.

Experimental validation of the optimized conditions

In order to confirm the model accuracy and the results from the response surface analysis, three additional experiments were performed randomly with the optimal medium compositions. Table V shows the yields of schizophyllan before and after optimization. Significant difference ($p < 0.05$) is obtained. The results verify the previous model that glucose at 18 g/l, yeast extract at 0.5 g/l, NH_4NO_3 at 0.48 g/l, and $MgSO_4$ at 0.05 g/l are the best combination for obtaining the maximum schizophyllan production.

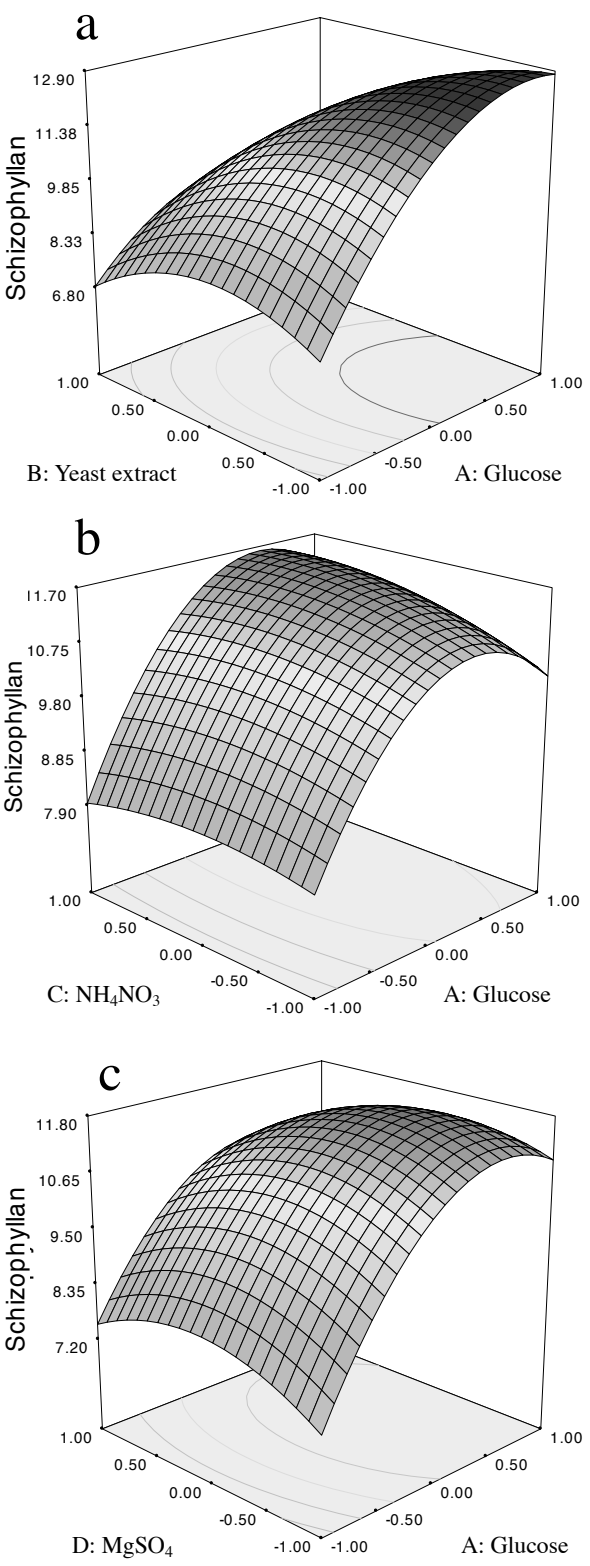

Fig. 3. Response surface curves of schizophyllan production by *S. commune*: the interaction between (a) glucose and yeast extract, (b) glucose and NH_4NO_3, and (c) glucose and $MgSO_4$.

Table V. Optimized medium composition for schizophyllan production by *S. commune*.

Serial no.	Component concentration [g/l]				Schizophyllan[e] [g/l]
	Glucose	Yeast extract	NH_4NO_3	$MgSO_4$	
1[a]	12.0	1.0	0.0	0.1	9.57 ± 0.68
2[b]	14.0	0.7	0.3	0.15	11.54 ± 0.12
3[c]	18.0	0.5	0.48	0.05	12.65 ± 0.75
4[d]	18.0	0.5	0.48	0.05	11.74 ± 0.00

[a] The values before optimization.
[b] The composition of centre point.
[c] The optimized values derived from RSM regression and schizophyllan yield in this study.
[d] The predicted optimum values and predicted maximal schizophyllan yield derived from RSM regression in this study.
[e] Results are means ± SD of three determinations with significant difference at $p < 0.05$.

Bioreactor fermentation results

The feasibility of the regression model in a 5-l scaled fermentor was also tested with the optimized medium. The maximum yield was 12.80 g/l with a biomass concentration of 22.90 g/l (DCW) after 7 d of cultivation.

In conclusion, statistically based experimental designs proved to be effective tools to optimize the medium components for maximal schizophyllan production. It was possible to determine optimal medium components using RSM to maximize the production of schizophyllan by *S. commune* from an initial value of 9.57 g/l to 12.80 g/l.

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