Dyes and Pigments 82 (2009) 142–146

Contents lists available at ScienceDirect

Dyes and Pigments

journal homepage: www.elsevier.com/locate/dyepig

The production of hypocrellin colorants by submerged cultivation of the medicinal fungus *Shiraia bambusicola*

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ARTICLE INFO

Article history:
Received 30 July 2008
Received in revised form
13 December 2008
Accepted 15 December 2008
Available online 3 January 2009

Keywords:
Hypocrellin
Central composite experimental design
Response surface analysis
Shiraia bambusicola
Submerged cultivation

ABSTRACT

Hypocrellin production using submerged cultivation of the medicinal fungus *Shiraia bambusicola* revealed that both glucose and $(NH_4)_2SO_4$ were optimal carbon and nitrogen sources. Hypocrellin production increased with increasing initial glucose concentration within the range of 10–50 g l^{-1} and $(NH_4)_2SO_4$ concentration in the range of 1–2 g l^{-1}. The effects of carbon and nitrogen concentration were optimized using central composite experimental design and response surface analysis; maximum hypocrellin production (196.94 ± 6.93 mg l^{-1}) was achieved using 45.7 g l^{-1} glucose and 1.93 g l^{-1} $(NH_4)_2SO_4$.

1. Introduction

Medicinal fungi have long been used as medicines in China and modern scientific research has shown that medicinal fungi are abundant sources of a wide range of useful natural products. *Shiraia bambusicola* P. Hennings is a parasitic fungus of bamboo, which grows primarily in the southern provinces of China. The stromata of *S. bambusicola* are commonly used in Chinese folk medicine to treat a variety of disorders in humans. A series of perylenequinoid pigments, including hypocrellin A (HA) and hypocrellin B (HB) have been isolated from the stromata of *S. bambusicola*; such hypocrellins show excellent light-induced antitumor and antiviral activities [1–6]. Previous research has also reported a series of other activities related to hypocrellins, such as the treatment of some vascular diseases [7].

Since their first isolation in the 1980s [8], hypocrellins have received intense interest in the context of photodynamic therapy (PDT) because of their wide absorption band in the visible region and extremely high singlet oxygen ($^1O_2$) generation ability [9–12]. Hypocrellins are the metabolites of several parasitic ascomycetes, including *Hypocrella bambusae* and *S. bambusicola.* However, as sources of the natural stromata of *S. bambusicola* or *H. bambusae* are limited, there is the need for an alternative, synthetic chemical or biological process for the preparation of hypocrellin. Although the synthesis of a key precursor compound, 4,9-dihydroxy-3,10-perylenequinone, has been described [13] the dimerization product of 1,2-naphthoquinone is a rate-limiting step [14,15]. However, hypocrellin has been biosynthesized successfully by the fermentation of *S. bambusicola* [16–19].

Recently, a new strain of *S. bambusicola* was isolated from the stromata of *S. bambusicola*. The purpose of this particular study is to optimize the submerged culture conditions required to produce hypocrellins using *S. bambusicola* with respect to carbon and nitrogen sources, with a view to obtain information that will be useful for hypocrellin production on a large scale.

2. Materials and methods

2.1. Microorganism

S. bambusicola UV-62 was screened and preserved by the Laboratory of Fermentation Engineering, Wenzhou University. The stock culture was maintained by bimonthly subcultivation on potato-agar-dextrose slants which were incubated at 25 °C for 5 days and then stored at 4 °C.

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doi:10.1016/j.dyepig.2008.12.012

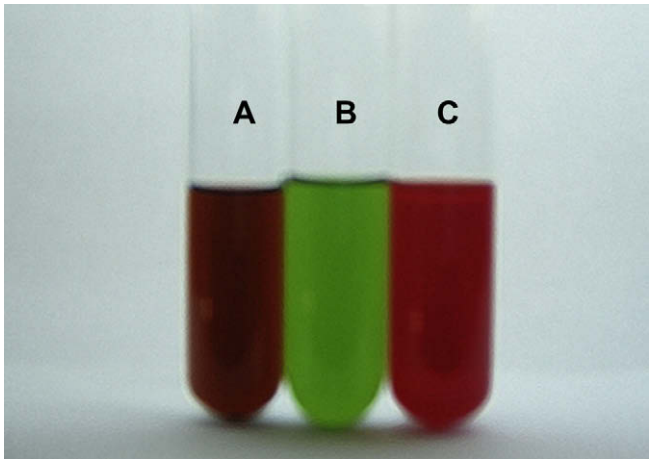

Fig. 1. Color reaction of the pigments produced by *S. bambusicola* UV-62. A, pigment acetone extract with $FeCl_3$ solution; B, pigment acetone extract with sodium hydroxide solution; C, pigment acetone extract.

2.2. Cultivation

Actively growing mycelia were obtained from a newly prepared agar-plate culture after it was incubated for 5 days at 25 °C. Around 0.3 cm × 0.3 cm sections of the mycelia were then transferred into a 500 ml flask that contained 100 ml of media, which consisted of the following components ($g\,l^{-1}$): K_2HPO_4, 1.0; $MgSO_4{\cdot}7H_2O$, 0.5; KCl, 0.5 and various carbon and nitrogen sources according to the experimental design.

Effects of carbon sources on liquid cultures of *S. bambusicola* were studied by using 30 $g\,l^{-1}$ of glucose, lactose, maltose, sucrose, mannitol, xylose as well as fructose. The other culture medium components were 3 g $(NH_4)_2SO_4\,l^{-1}$, 1 g $K_2HPO_4\,l^{-1}$, 0.5 g $MgSO_4{\cdot}7H_2O\,l^{-1}$, and 0.5 g $KCl\,l^{-1}$. The effect of nitrogen source on the fungus culture was studied by using 3 $g\,l^{-1}$ of yeast extract, peptone, urea, $(NH_4)_2SO_4$, $NaNO_3$, casein as well as NH_4NO_3. The other culture medium components were 30 g glucose l^{-1}, 1 g $K_2HPO_4\,l^{-1}$, 0.5 g $MgSO_4{\cdot}7H_2O\,l^{-1}$, and 0.5 g $KCl\,l^{-1}$. For the investigation on initial glucose and $(NH_4)_2SO_4$ concentrations, glucose was used with levels of 10, 20, 30, 40, 50 and 60 $g\,l^{-1}$ and $(NH_4)_2SO_4$ was used with levels of 1, 2, 3, 4, 5 and 6 $g\,l^{-1}$. In experiments on the effects of carbon/nitrogen ratios, the levels of glucose and $(NH_4)_2SO_4$ in the medium were changed and a statistical approach was applied.

The media were autoclaved at 121 °C for 20 min, where carbon source was separately sterilized. Unless otherwise specified, the cultivation was carried out at 25 °C on a rotary shaker incubator at 175 rpm for 5 days. In all experiments, multiple flasks were run at the same time, and three flasks were sacrificed at each sampling point.

2.3. Central composite experimental design (CCED)

For determining the optimal nutritional conditions for hypocrellin production, CCED was conducted to locate the true optimum concentrations of glucose and $(NH_4)_2SO_4$. The levels of variables for CCED experiments were selected according to the results of a one-variable-at-a-time strategy. For predicting the optimum point, a second-order polynomial equation was fitted to correlate relationship between independent variables (glucose and $(NH_4)_2SO_4$) and response (hypocrellin yield) by a multiple regression technique. The corresponding equation is:

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ii} X_i^2 + \Sigma\beta_{ij} X_i X_j \quad (1)$$

where, β_0 is the constant, β_i is the linear coefficient, β_{ii} is the quadratic coefficient and $\beta_{i,j}$ the cross product coefficient. X_i and X_j are the levels of the independent variable. The fitness of the model was evaluated by the correlation coefficient R^2 and the statistical significance was determined by an *F*-test. The statistical analyses were performed using the SAS 8.0 program (SAS Institute Inc., NC, USA).

2.4. Analytical methods

After the cultivation was terminated, mycelia were collected by centrifugation at 5000 × g for 20 min, and washed with distilled water three times, then dried. The concentration of fungal biomass was determined gravimetrically.

The determination of hypocrellin content was similar to the method described by Liu et al. [20]. The dried mycelia (100 mg) were treated in 6 M HCl at 60 °C (1 h), and the precipitate was collected by centrifugation and extracted by acetone (5 ml) for 24 h (twice). After removal of mycelia by centrifugation, the content of hypocrellin in supernatant was measured by a spectrophotometer at 465 nm.

3. Results and discussion

3.1. Identification of hypocrellins

Fig. 1 showed the pigments produced by *S. bambusicola* UV-62 which became green under alkaline condition and turned dark

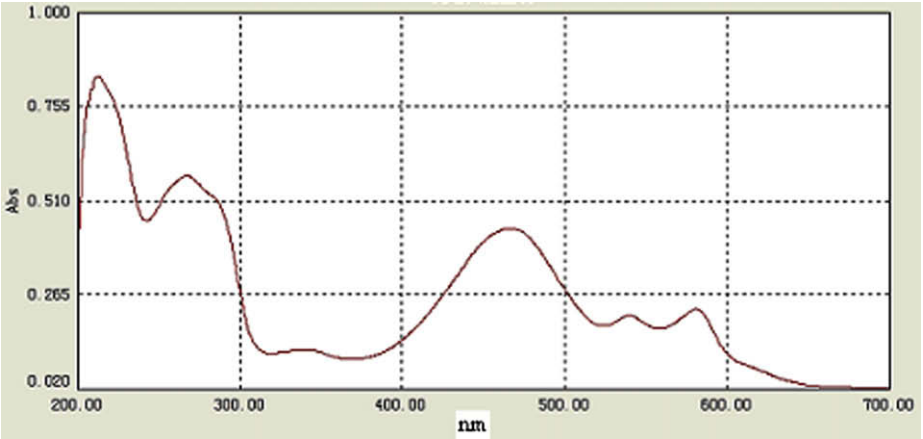

Fig. 2. UV–vis absorbing spectrum of the main pigment component produced by *S. bambusicola* UV-62.

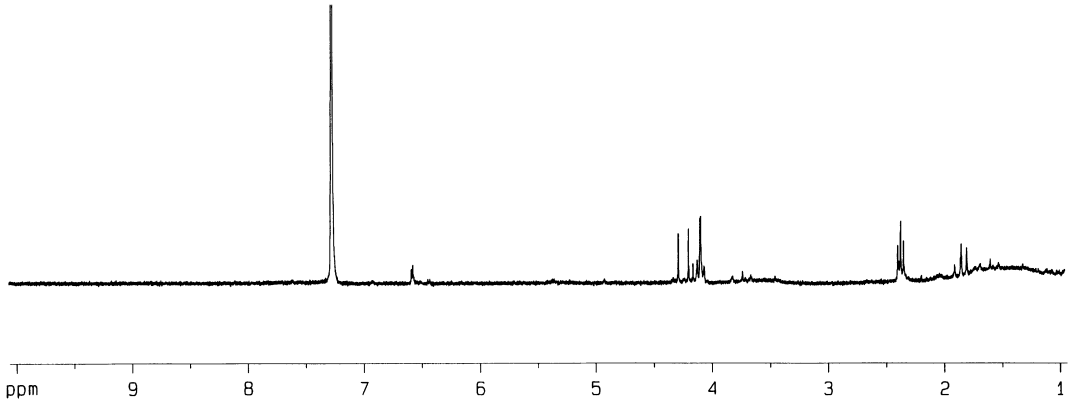

Fig. 3. The ^{1}H NMR (300 MHz, $CDCl_3$) spectrum of the main pigment component produced by *S. bambusicola* UV-62.

Table 1
Effects of carbon sources on the mycelial growth and hypocrellin production of *S. bambusicola*.[a]

Carbon sources (30 g l^{-1})	Biomass (g l^{-1})	Hypocrellin production (mg l^{-1})
Glucose	9.63 ± 0.15	162.80 ± 5.22
Lactose	0.74 ± 0.05	–
Maltose	5.45 ± 0.36	25.11 ± 1.93
Sucrose	11.65 ± 0.24	129.02 ± 2.71
Mannitol	9.29 ± 0.44	48.40 ± 2.56
Xylose	8.71 ± 0.41	110.06 ± 2.62
Fructose	8.43 ± 0.23	120.71 ± 3.07

[a] Cells were cultivated at 25 °C for 5 d on a rotary shaker at 175 rpm in the media containing (g l^{-1}) $(NH_4)_2SO_4$ 3, K_2HPO_4 1.0, $MgSO_4{\cdot}7H_2O$ 0.5, KCl 0.5 with various carbon sources, respectively.

Table 2
Effects of nitrogen sources on the mycelial growth and hypocrellin production of *S. bambusicola*.[a]

Nitrogen sources (3 g l^{-1})	Biomass (g l^{-1})	Hypocrellin production (mg l^{-1})
Yeast extract	12.77 ± 0.69	63.98 ± 2.63
Peptone	10.19 ± 0.20	97.17 ± 4.76
Urea	7.99 ± 0.58	5.85 ± 0.32
$(NH_4)_2SO_4$	9.72 ± 0.41	163.06 ± 4.71
$NaNO_3$	10.88 ± 0.75	18.75 ± 0.87
Casein	10.48 ± 0.18	153.92 ± 5.99
NH_4NO_3	9.07 ± 0.37	28.40 ± 1.28

[a] Cells were cultivated at 25 °C for 5 d on a rotary shaker at 175 rpm in the media containing (g l^{-1}) Glucose 30, K_2HPO_4 1.0, $MgSO_4{\cdot}7H_2O$ 0.5, KCl 0.5 with various nitrogen sources, respectively.

purple when $FeCl_3$ was added, that were the typical color reaction of hypocrellin [8]. Furthermore, the pigments were chromatographed over silica gel using the elution of $CHCl_3$/MeOH (100:1), and the main component was collected. Compared with HA standard, the main component had the same R_f value by thin-layer chromatography (TLC) on silica gel. UV–vis absorption curve (Fig. 2) showed that the main component had four absorption peaks at 581.5 nm, 540 nm, 466.5 nm, 267.5 nm, and 212.5 nm, and that were the typical UV–vis absorption spectrum of HA [21]. ^{1}H NMR spectra (Fig. 3) of the main component were (300 MHz, $CDCl_3$; δ): 1.705 (3H, s, H-16), 1.843 (3H, s, H-18), 2.384 (1H, d, H_B-13), 3.724 (1H, d, H_A-13), 4.051 (3H, s, 7- or 6-OCH_3), 4.061 (3H, s, 6- or 7-OCH_3), 4.083 (3H, s, 11- or 2-OCH_3), 4.114 (3H, s, 2- or 11-OCH_3), 6.57 (1H, s, H-8 or H-5), 6.58 (1H, s, H-5 or H-8). Based on the data of ^{1}H NMR, color reaction and UV–vis absorbing spectrum and compared with HA standard on TLC, the main component of pigments produced by *S. bambusicola* UV-62 was confirmed as HA.

3.2. Effect of carbon and nitrogen sources

Culture medium is important to the yield of any fermentation products, and carbon and nitrogen sources generally play a significant role because these nutrients are directly linked with cell proliferation and metabolite biosynthesis [22,23]. Carbohydrates are a major component of the cytoskeleton and an important nutritional requirement for the growth and development of higher fungi [24]. To identify a suitable carbon source for hypocrellin production by submerged cultivation of *S. bambusicola*, different carbohydrates, i.e. glucose, lactose, maltose, sucrose, mannitol, xylose, and fructose were tested. As shown in Table 1, among the sources examined, sucrose yielded the best mycelial growth (11.65 g l^{-1}). A similar result was reported in *Oudemansiella radicata* [23]. Following sucrose, glucose and mannitol were suitable carbon sources for the growth of *S. bambusicola*, but *S. bambusicola* could not grow in lactose medium. As to hypocrellin production, glucose (162.80 mg l^{-1}) was the best carbon source followed by sucrose (129.02 mg l^{-1}), fructose (120.71 mg l^{-1}) and xylose (110.06 mg l^{-1}), respectively.

Table 3
Effects of initial glucose concentration on the mycelial growth and hypocrellin production of *S. bambusicola*.[a]

Glucose concentration (g l^{-1})	Biomass (g l^{-1})	Hypocrellin production (mg l^{-1})
10	3.57 ± 0.12	34.71 ± 1.27
20	5.88 ± 0.50	48.91 ± 2.63
30	9.87 ± 0.57	162.77 ± 6.12
40	10.18 ± 0.75	185.17 ± 2.82
50	13.68 ± 0.29	160.01 ± 6.23
60	12.39 ± 0.14	148.73 ± 7.10

[a] Cells were cultivated at 25 °C for 5 d on a rotary shaker at 175 rpm in the media containing (g l^{-1}) $(NH_4)_2SO_4$ 3, K_2HPO_4 1.0, $MgSO_4{\cdot}7H_2O$ 0.5, KCl 0.5 with different glucose concentration, respectively.

Table 4
Effects of initial $(NH_4)_2SO_4$ concentration on the mycelial growth and hypocrellin production of *S. bambusicola*.[a]

$(NH_4)_2SO_4$ concentration (g l^{-1})	Biomass (g l^{-1})	Hypocrellin production (mg l^{-1})
1	5.96 ± 0.37	144.47 ± 5.76
2	8.89 ± 0.41	201.56 ± 5.69
3	10.12 ± 0.39	172.71 ± 6.57
4	10.82 ± 0.65	58.52 ± 3.92
5	12.09 ± 0.31	50.78 ± 3.74
6	11.79 ± 0.26	58.04 ± 5.71

[a] Cells were cultivated at 25 °C for 5 d on a rotary shaker at 175 rpm in the media containing (g l^{-1}) glucose 40, K_2HPO_4 1.0, $MgSO_4{\cdot}7H_2O$ 0.5, KCl 0.5 with different $(NH_4)_2SO_4$ concentration, respectively.

Table 5
Experimental design and responses of central composite experimental design (CCED).

Runs	X_2 glucose ($g\,l^{-1}$)	$X_2(NH_4)_2SO_4$ ($g\,l^{-1}$)	Biomass ($g\,l^{-1}$) Observed	Hypocrellin ($mg\,l^{-1}$) observed
1	30 (−1)	1 (−1)	7.35 ± 0.12	169.02 ± 7.11
2	30 (−1)	3 (1)	15.25 ± 0.34	163.49 ± 5.77
3	60 (1)	1 (−1)	7.19 ± 0.47	172.23 ± 8.98
4	60 (1)	3 (1)	15.19 ± 0.81	149.64 ± 4.54
5	23.79 (−1.414)	2 (0)	7.52 ± 0.14	169.76 ± 9.11
6	66.21 (1.414)	2 (0)	12.67 ± 0.41	180.38 ± 9.09
7	45 (0)	0.586 (−1.414)	4.88 ± 0.23	133.80 ± 2.54
8	45 (0)	3.414 (1.414)	12.66 ± 0.44	129.04 ± 1.79
9	45 (0)	2 (0)	11.09 ± 0.37	194.94 ± 5.95
10	45 (0)	2 (0)	11.26 ± 0.40	201.91 ± 8.13
11	45 (0)	2 (0)	10.92 ± 0.22	199.46 ± 6.53
12	45 (0)	2 (0)	11.18 ± 0.25	192.26 ± 4.71
13	45 (0)	2 (0)	10.94 ± 0.12	203.58 ± 6.26

Different nitrogen sources, i.e. yeast extract, peptone, urea, $(NH_4)_2SO_4$, $NaNO_3$, casein, and NH_4NO_3 were used to identify a suitable nitrogen source. The results are shown in Table 2. Among the various nitrogen sources, yeast extract ($12.77\,g\,l^{-1}$) yielded the highest mycelial growth followed by $NaNO_3$ ($10.88\,g\,l^{-1}$), casein ($10.48\,g\,l^{-1}$) and peptone ($10.19\,g\,l^{-1}$). However, $(NH_4)_2SO_4$ ($163.06\,mg\,l^{-1}$)was the best nitrogen source for hypocrellin production, and did not differ significantly from casein ($153.92\,mg\,l^{-1}$). From the above data, it was apparent that glucose and $(NH_4)_2SO_4$ were most beneficial to hypocrellin production by submerged cultivation of *S. bambusicola*.

3.3. Effect of initial glucose and $(NH_4)_2SO_4$ concentrations

Based on the above results, glucose and $(NH_4)_2SO_4$ were selected as carbon and nitrogen sources for further studies. The effects of initial sucrose concentrations on mycelial growth and hypocrellin production were shown in Table 3. Mycelial growth increased with an increase of initial glucose concentrations between 10 and $50\,g\,l^{-1}$. When the initial glucose concentration was more than $50\,g\,l^{-1}$, the mycelial growth decreased. For hypocrellin formation, its highest production was obtained at $40\,g\,l^{-1}$ initial glucose, which reached $185.17 \pm 2.82\,mg\,l^{-1}$. The results indicated that a high initial glucose concentration ($50\,g\,l^{-1}$) was unfavorable to hypocrellin biosynthesis.

The effects of initial $(NH_4)_2SO_4$ concentrations on mycelial growth and hypocrellin production are shown in Table 4. Mycelial growth increased with an increase of initial $(NH_4)_2SO_4$ concentrations between 1 and $5\,g\,l^{-1}$, whereas maximal hypocrellin production ($201.56 \pm 5.69\,mg\,l^{-1}$) was obtained at $2\,g\,l^{-1}$ initial $(NH_4)_2SO_4$. The hypocrellin production decreased when initial $(NH_4)_2SO_4$ concentration was over $2\,g\,l^{-1}$. The results indicated that a high initial $(NH_4)_2SO_4$ concentration was favourable to mycelial growth, but inhibited hypocrellin formation by submerged cultivation of *S. bambusicola*.

3.4. Optimization of carbon/nitrogen concentration

The concentrations of both carbon and nitrogen sources and their balance in medium are very important for optimal metabolite production [23]. To investigate the combined effect of carbon and nitrogen sources, the statistical approach of CCED was used to identify and quantify the interaction between initial glucose and $(NH_4)_2SO_4$ concentration. The levels of variables for CCED experiments were selected according to the above results of one-at-a-time strategy. Table 5 showed the detailed experimental design and results. From the observations, a regression equation (second-order polynomial), which was an empirical relationship between hypocrellin production and the test variables in coded units was got by the application of RSM and that was given as follows:

$$Y = 198.4287 + 0.54721X_1 - 4.357X_2 - 9.0912X_1^2 - 4.265X_1X_2 - 30.9228X_2^2 \quad (2)$$

The statistical analysis indicated that the proposed model was adequate with very satisfactory value of regression coefficient ($R^2 = 0.9408$) for the response and the probability (p) value of the regression model was less than 0.0004. R^2 was defined as the ratio of the explained variation to the total variation and was a measure of the degree of fit [25]. The closer the value of R^2 approached unity, the better the empirical models fits the actual data [26]. The response surface plot obtained from Equation (2) is shown in Fig. 4. It is evident that hyrocrellin production reached its maximum at a combination of coded level 0.047 (X_1, glucose) and −0.073(X_2, $(NH_4)_2SO_4$) by canonical analysis of SAS software. The model predicted a maximum response of 198.6 $mg\,l^{-1}$ hypocrellin at levels of $45.7\,g\,l^{-1}$ glucose and $1.93\,g\,l^{-1}$ $(NH_4)_2SO_4$ as optimized medium components.

The adequacy of the model equation for predicting the optimum response values was tested using $45.7\,g\,l^{-1}$ glucose and $1.93\,g\,l^{-1}$ $(NH_4)_2SO_4$ as initial carbon and nitrogen sources. This set of carbon/nitrogen concentration was determined to be optimum by the RSM optimization approach and was also used to validate experimentally and predict the value of the responses using the model equation. Good agreement existed between values calculated using the model equation and the experimental value of the response

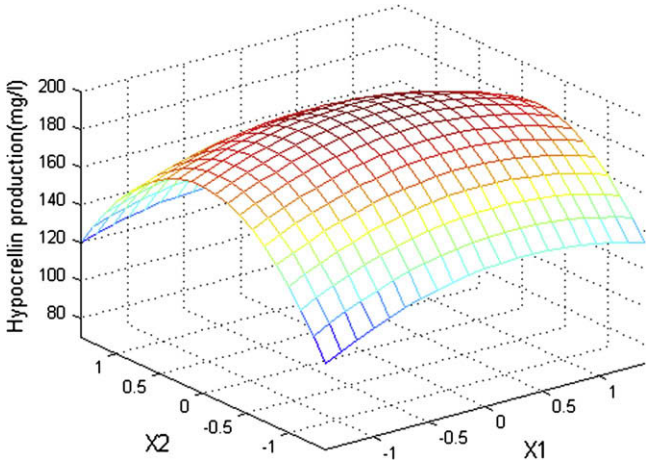

Fig. 4. Response surface plot showing the combined effect of glucose (X_1) and $(NH_4)_2SO_4$ (X_2) on hypocrellin production (Y) by *S. bambusicola*.

Table 6
Predicted and experimental value of response at the optimal carbon/nitrogen concentration for hypocrellin production.

Response	Predicted value	Experimental value[a]	Range
Hypocrellin production ($mg\,l^{-1}$)	198.6023	196.94 ± 6.93	185.92–204.57

[a] Mean value of five times.

variables and the experimental results were found to be in agreement with the predicted ones (Table 6).

4. Conclusion

In this work, a process of submerged cultivation of *S. bambusicola* for hypocrellin production was demonstrated. The effects of major nutrients, i.e. carbon and nitrogen sources and carbon/nitrogen ratios, on hypocrellin production were studied in order to obtain a suitable fermentation medium, and glucose and $(NH_4)_2SO_4$ were optimal carbon and nitrogen sources for hypocrellin production, respectively. According to the central composite design and response surface analysis, an optimal carbon source ($45.7\,g\,l^{-1}$ glucose) and nitrogen source ($1.93\,g\,l^{-1}$ $(NH_4)_2SO_4$) were identified and a maximal hypocrellin production $196.94 \pm 6.93\,mg\,l^{-1}$ was successfully obtained in same cultivation conditions. The fundamental information obtained in this work is beneficial for further development of *S. bambusicola* cultivation process for production of hypocrellin on a large scale.

Acknowledgement

Financial support was received from Zhejiang Department of Science and Technology (Contract No. 2007C23012), PR China.

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