

Research Article

Optimization of Large-Scale Culture Conditions for the Production of Cordycepin with *Cordyceps militaris* by Liquid Static Culture

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Cordycepin is one of the most important bioactive compounds produced by species of *Cordyceps sensu lato*, but it is hard to produce large amounts of this substance in industrial production. In this work, single factor design, Plackett-Burman design, and central composite design were employed to establish the key factors and identify optimal culture conditions which improved cordycepin production. Using these culture conditions, a maximum production of cordycepin was 2008.48 mg/L for 700 mL working volume in the 1000 mL glass jars and total content of cordycepin reached 1405.94 mg/bottle. This method provides an effective way for increasing the cordycepin production at a large scale. The strategies used in this study could have a wide application in other fermentation processes.

1. Introduction

Cordyceps militaris is an entomopathogenic fungus belonging to Ascomycota, Sordariomycetidae, Hypocreales, and Cordycipitaceae [1] and is one of the most important traditional Chinese medicinal mushrooms. *Cordyceps militaris* is the type species of *Cordyceps*, which internally parasitizes larva or pupa of lepidopteran insects and forms fruiting bodies on their insect hosts. *Cordyceps militaris* has long been recognized as a desirable alternative for natural *Ophiocordyceps sinensis* [2] as it has been given Chinese Licence number Z20030034/35. This is because the gathering of *Ophiocordyceps sinensis* is causing substantial reductions in populations [3]. *Cordyceps militaris* produces many bioactive compounds, including polysaccharides, cordycepin, adenosine, amino acid, organic selenium, ergosterol, sterols, cordycepic acid, superoxide dismutase (SOD), and multivitamins [4, 5].

Cordycepin (3'-deoxyadenosine), a nucleoside analog, was first isolated from *C. militaris* [6] and is one of the species most important biologically active metabolites. It has been regarded as a medicinal agent responsible for immunological regulation [7], anticancer [8], antifungus [9], antiviral [10], antileukemia [11, 12], and antihyperlipidemia [13] activities. Cordycepin is also a Phase I/II clinical stage drug candidate for treatment of refractory acute lymphoblastic leukemia (ALL) patients who express the enzyme terminal deoxynucleotidyl transferase (TdT) (<http://www.ClinicalTrials.gov> verified by OncoVista, Inc., 2009).

In previous work, cordycepin has been synthesized by chemical [14, 15] and microbial fermentation using *C. militaris* [6] or *Aspergillus nidulans* [16, 17]. Solid-state fermentation [18, 19], submerged culture [4, 20–24], and surface liquid culture [25–27] have been used in microbial fermentation of cordycepin. Cordycepin obtained through chemistry

pathways is hard to purify, and the cost is much higher than through biology fermentation. Thus a major need is to improve the biology methodology [28]. Fermentation time is too long and is difficult to achieve large scale production via solid-state fermentation [18, 19]. Productivity is generally low, the costs are high, and fermentation processes are easily contaminated in submerged culture in large fermenters [4, 20, 21, 29]. Productivity in surface culture techniques is higher as compared to other methods [29, 30] and the cost is lower [23]. New technologies, such as space mutation treatment and high-energy ion beam irradiation, have been used to obtain better Cordycepin producing, novel mutants of *C. militaris*. The resulting mutants were higher cordycepin produces, than the wild strain [30, 31]. Bu et al. [20] reported that the cordycepin in *C. militaris* was substantially increased by the elicitor of *Phytophthora* sp. Research result showed that glucose and yeast extract were effective media components for improved cordycepin production by *C. militaris* [32, 33]. There have been other studies using different culture conditions [21, 24, 25, 32], culture medium, and additives [4, 22–24, 26, 27] for the production of cordycepin via liquid culture. However, as far as we know, these reports studied cordycepin production in 250 mL or 500 mL Erlenmeyer flasks, and there have been no reports to improved cordycepin production using static liquid culture in 1000 mL glass jars. The latter process is a good way to scale up large scale cordycepin production from the laboratory to industry.

In this study, the effects of working volume, carbon sources, nitrogen sources, inorganic salts, growth factor, nucleoside analogue, and amino acid additions were studied in order to improve the cordycepin production by static liquid culture of *C. militaris* (strain CGMCC2459) in 1000 mL glass jars. The results suggested that the optimization medium conditions were helpful for improved large scale cordycepin production.

2. Materials and Methods

2.1. Microorganism and Seed Culture. The isolate of *C. militaris* (strain CGMCC2459) used in the present study was collected from Mt. Qingcheng in Sichuan Province, China. The microorganism was maintained on potato dextrose agar (PDA) slants. Slants were incubated at 25°C for 7 days and then stored at 4°C. The seed culture was grown in a 250 mL flask containing 70 mL of basal medium (sucrose 20 g/L; peptone 20 g/L; KH_2PO_4 1 g/L; and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g/L) at 25°C on a rotary shaker incubator at 150 rev/min for 5 days [24].

2.2. Basal Medium and Static Culture of Glass Jars. The basal medium composition for the fermentation was as follows: sucrose 20 g/L; peptone 20 g/L; KH_2PO_4 1 g/L; and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g/L. The pH was not adjusted, followed by autoclaving for 30 min on the 121°C. The static culture experiments were performed in 1000 mL glass jars (inner diameter 110 mm, height 150 mm) containing basal medium after inoculating with 10% (v/v; the biomass dry weight of seed culture is 54 mg/mL) of the seed culture. The culture was incubated at 25°C without moving for 35 days, and samples were collected at the end of the fermentation from the

glass jars for analyzing biomass dry weight and cordycepin production.

2.3. Static Culture Conditions. The effects of factors affecting cell growth and the production of cordycepin by *C. militaris* were studied using a one-factor-at-a-time method for static culture. The effects of carbon sources on cordycepin production were studied by substituting carbon sources such as sucrose, lactose, soluble starch, and dextrin for glucose at 25°C for 35 days. Effects of nitrogen sources (yeast extract, beef extract, NH_4NO_3 , NaNO_3 , NH_4Cl , casein, and carbamide) and inorganic salts ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, KCl, ZnSO_4 , $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, and $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$) were also studied using static culture. Growth factors (Vitamin B_1 (VB_1), Vitamin B_6 (VB_6), Vitamin B_7 (VB_7), Vitamin B_{11} (VB_{11}), α -naphthylacetic acid (NAA), 3-Indoleacetic acid (IAA), and 2,4-dichlorophenoxyacetic acid (2,4-D)) were supplemented for 10 mg/L in basal media. Nucleoside analogues (1 g/L) and amino acids (8 g/L) established in our previous study [23] as an initial concentration were separately added to the optimal concentration of carbon and nitrogen source, inorganic salts, and growth factors and cultivated at 25°C for 35 days. All experiments were carried out at triplicate, and mean of results is presented.

2.4. Analytical Methods. Samples collected at 35 days from the glass jars were centrifuged at 2810 \times g for 20 min. The mycelium at the bottom of tubes was washed sufficiently with a large amount of distilled water and dried to a constant dry weight at 55°C.

For analysis of extracellular cordycepin, the resulting culture filtrate was obtained by centrifugation at 2810 \times g for 20 min. The supernatant was filtered through a 0.45 μm membrane and the filtrate was analyzed by HPLC (1100 series, Agilent Technology, USA). Accurate quantities of cordycepin (Sigma, USA) were dissolved in distilled water, to give various concentrations for calibration. The mobile phase was 10 mmol/L KH_2PO_4 , which was dissolved in methanol/distilled water (6 : 94). Elution was performed at a flow rate of 1.0 mL/min with column temperature at 45°C and UV wavelength of 259 nm. Mean values were computed from triplicate samples.

2.5. Plackett-Burman Design. The Plackett-Burman design, an effective technique for medium-component optimization [35, 36], was used to select factors that significantly influenced hydrogen production. Sucrose (X_1), peptone (X_2), $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ (X_4), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (X_5), and VB_1 (X_6) were investigated as key ingredients affecting cordycepin production. Based on the Plackett-Burman design, a 15-run was applied to evaluate eleven factors (including two virtual variables). Each factor was prepared in two levels: -1 for low level and +1 for high level. Table 1 illustrates the variables and their corresponding levels used in the experimental design. The values of two levels were set according to our preliminary experimental results. The Plackett-Burman design and the response value of cordycepin production are shown in Table 2.

TABLE 1: Range of different factors investigated with Plackett-Burman design.

Symbol	Variables	Experimental value	
		Low (-1)	High (+1)
X_1	Sucrose (g/L)	20	25
X_2	Peptone (g/L)	20	25
X_3	Virtual 1	-1	1
X_4	$K_2HPO_4 \cdot 3H_2O$ (g/L)	1	1.25
X_5	VB_1 (g/L)	10	12.5
X_6	$MgSO_4 \cdot 7H_2O$ (g/L)	1	1.25
X_7	Virtual 2	-1	1

2.6. *Response Surface Methodology.* Response surface methodology using a central composite design was applied to batch cultures of *C. militaris*, for identifying the effects of process variables [35, 36]. In this study, the basic nutrient (carbon sources, nitrogen sources, inorganic salts, and growth factors) and additives (amino acid, nucleoside analogue) were studied for cordycepin production using static liquid culture. In the first test, a three-factor, five-level central composite design with 20 runs was employed. Tested variables (sucrose, $K_2HPO_4 \cdot 3H_2O$, and $MgSO_4 \cdot 7H_2O$) were denoted as X_1 , X_4 , and X_6 , respectively, and each of them was assessed at five different levels, combining factorial points (-1, -1), axial points (-1.6818, +1.6818), and central point (0), as shown in Table 3. Based on the above results, another test, a three-factor, five-level central composite design with 20 runs was employed. Tested variables (amino acid, nucleoside analogue, and culture time) were denoted as A, B, and C, respectively, and each of them was assessed at five different levels, combining factorial points (-1, +1), axial points (-1.6818, +1.6818), and central point (0), as shown in Table 4.

2.7. *Statistical Analysis.* Dry weight and cordycepin production are expressed as means \pm SD. An analysis of variance (ANOVA) followed by Tukey’s test was applied for multiple comparisons of significant analyses at $P < 0.05$. Statistical data analyses were performed in SPSS version 17.0 software packet. Design-Expert Version 8.0.5b software package (Stat-Ease Inc., Minneapolis, USA) was used for designing experiments as well as for regression and graphical analysis of the experimental data obtained.

3. Results and Discussion

3.1. *Effects of Working Volume on the Biomass and Cordycepin Production.* Dissolved oxygen concentration is the key factor in the medium for cell growth and metabolite biosynthesis [21]. Dissolved oxygen does not only have an important function in the respiratory chain, but also in metabolite composition [37, 38]. A previous study showed that the highest cordycepin production and productivity were obtained at lower dissolved oxygen levels [21]. Masuda et al. [25] also reported that a lower medium depth was most efficient for cordycepin production in *C. militaris* by surface culture.

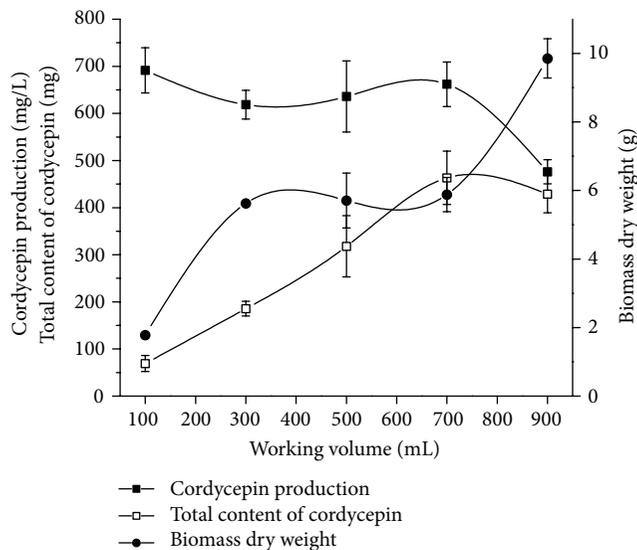


FIGURE 1: Effects of working volume on the production of cordycepin, total production of cordycepin, and biomass dry weight (total content of cordycepin (mg) = cordycepin production (mg/L) \times working volume (mL)).

In this study, we tried to establish the most efficient working volume of medium for improved cordycepin production. Cultures of *C. militaris* were prepared at the working volumes of 100 to 900 mL (corresponding to a medium depth of 1.26 to 11.31 cm). As shown in Figure 1, cordycepin production reduced gradually with increasing working volume of the medium, from 100 to 700 mL. However, there was no significant difference in cordycepin production in different working volumes. Obviously, the highest working volume of 900 mL did not help in cordycepin production. The result indicates that there is an upper dissolved oxygen limit in the medium for cordycepin production [21]. Lower working volumes result in higher cordycepin productivity, with the highest peak (463.33 ± 56.72 mg of cordycepin) produced at using 700 mL of media. Changes in biomass values were small (between 300 and 700 mL) because of the restricted area and thickness of the mycelial mat. In order to obtain higher cordycepin production, the most effective medium amount was 700 mL (corresponding to an 8.8 cm medium depth) and used as the media volume for next experiment.

3.2. *Effects of Carbon and Nitrogen Sources on Cordycepin Production.* To find a suitable carbon source for *C. militaris* cordycepin production we added various carbon sources at a concentration of 20 g/L to the sugar-free basal medium. Glucose was previously found to be an excellent precursor of cordycepin production [39]. However, as shown in Figure 2(a), sucrose and lactose proved to be better carbon sources for cordycepin production than glucose in this study. Cordycepin production reached 843.63 ± 66.70 mg/L of sucrose and 823.72 ± 85.64 mg/L of lactose, respectively. Therefore, sucrose was selected as the main carbon source in the remaining experiment.

TABLE 2: Plackett-Burman design and response values.

Runs	Experimental value							Y (mg/L) Cordycepin production
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	
1	1	-1	1	-1	-1	-1	1	812.36 ± 26.83
2	1	1	-1	1	-1	-1	-1	1395.18 ± 8.4
3	-1	1	1	-1	1	-1	-1	900.25 ± 10.29
4	1	-1	1	1	-1	1	-1	802.45 ± 45.43
5	1	1	-1	1	1	-1	1	1097.66 ± 25.57
6	1	1	1	-1	1	1	-1	845.87 ± 24.94
7	-1	1	1	1	-1	1	1	786.35 ± 7.61
8	-1	-1	1	1	1	-1	1	805.08 ± 29.1
9	-1	-1	-1	1	1	1	-1	920.48 ± 16.21
10	1	-1	-1	-1	1	1	1	694.01 ± 79.51
11	-1	1	-1	-1	-1	1	1	497.28 ± 4.44
12	-1	-1	-1	-1	-1	-1	-1	592.83 ± 16.13
13	0	0	0	0	0	0	0	1134.14 ± 2.59
14	0	0	0	0	0	0	0	1100.21 ± 0.08
15	0	0	0	0	0	0	0	1133.56 ± 1.85

TABLE 3: Factors and levels of central composite design for carbon sources and inorganic salts.

Symbol	Variables	Code level				
		-1.6818	-1	0	1	1.6818
X_1	Sucrose (g/L)	3.1821	10	20	30	36.8179
X_4	$K_2HPO_4 \cdot 3H_2O$ (g/L)	0.1591	0.5	1	1.5	1.8409
X_6	$MgSO_4 \cdot 7H_2O$ (g/L)	0.1591	0.5	1	1.5	1.8409

In previous work, nitrogen showed a regulating role important in cordycepin production and had two effects [40]. One effect was negative since, in excess, *N* promoted a faster mycelial growth and consequently diverted the source of carbon toward energy and biomass production. The other effect was positive because a moderate input contributed to the maintenance of citric acid productive biomass [40]. To investigate the effect of nitrogen sources on cordycepin production in *C. militaris*, various compounds containing nitrogen (inorganic and organic nitrogen) were added individually to nitrogen free basal medium at a concentration of 20 g/L. Among the 8 nitrogen sources tested, peptone, yeast extract, beef extract, casein, and NH_4NO_3 were favorable to the cordycepin production (Figure 2(b)). Organic nitrogen was advantageous to both growth and biosynthesis of metabolites. The result is consistent with the experimental data reported [18] and showed that maximum cordycepin production resulted when the peptone was used as a nitrogen source.

3.3. Effects of Inorganic Salt and Growth Factor on the Cordycepin Production. Inorganic ion was one of the most important nutrition components of medium for the mycelial growth [41]. In order to investigate the effects of inorganic salt for the cordycepin production in *C. militaris*, we tested nine types (at 1 g/L) of inorganic salts (Figure 3(a)). Media with only 20 g/L glucose and 20 g/L peptone were used as the control. The

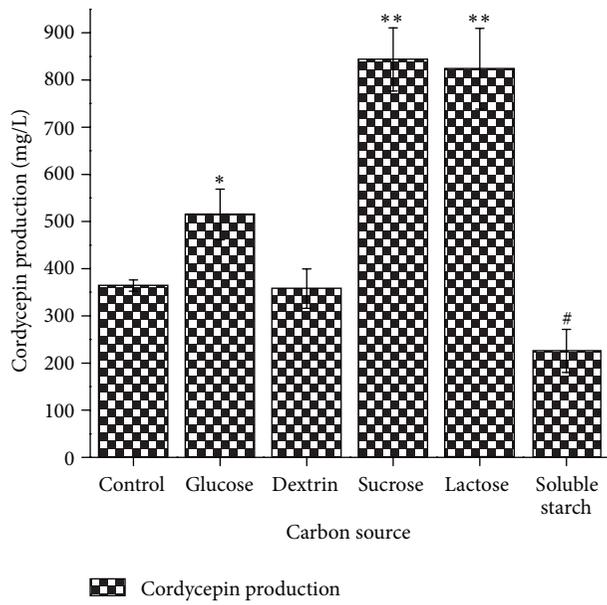
TABLE 4: Factors and levels of central composite design for amino acid, nucleoside analogue, and time.

Symbol	Variables	Code level				
		-1.6818	-1	0	1	1.6818
A	Hypoxanthine (g/L)	0.53	1	5	9	10.53
B	L-alanine (g/L)	5.27	8	12	16	18.72
C	Culture time (days)	-0.09	4	10	16	20.09

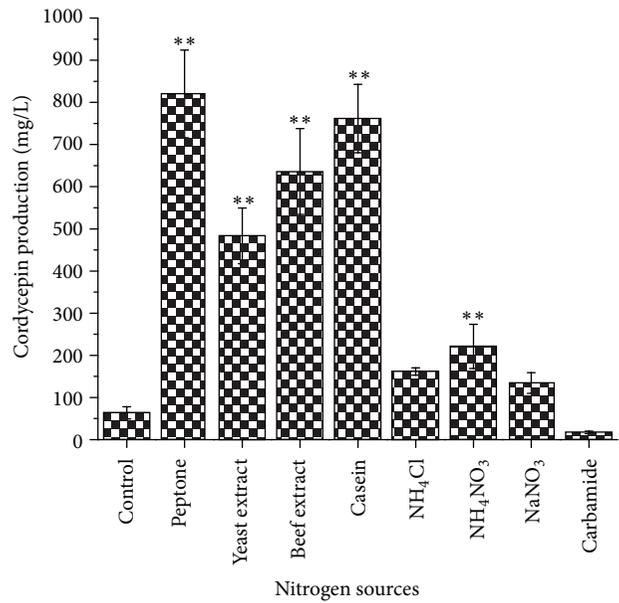
highest cordycepin production (1120.30 ± 105.28 mg/L) by *C. militaris* was observed in medium, when $K_2HPO_4 \cdot 3H_2O$ was used as an inorganic salt. KH_2PO_4 , $MgSO_4 \cdot 7H_2O$, KCl, and $MgCl_2 \cdot 6H_2O$ were also useful inorganic salts. At last, $MgSO_4 \cdot 7H_2O$ and $K_2HPO_4 \cdot 3H_2O$ were recognized as favorable bioelements for production of cordycepin.

Growth factor is essential for growth response and metabolite production [42]. In order to find the optimal growth factor for cordycepin production, *C. militaris* was cultured in a basal medium with different vitamins and plant growth hormones in static liquid culture. Cordycepin production increased in media with added 10 mg/L of VB_1 , NAA, and VB_{11} (Figure 3(b)). Maximum cordycepin production (1159.34 ± 109.01 mg/L) occurred when VB_1 was used as the growth factor.

3.4. Screening of Important Variables Using Plackett-Burman Design. The data (Table 2) indicated wide variation in cordycepin production in the 15 tests. The data suggested that process optimization is important for improving the efficiency of cordycepin production. Analysis of the regression coefficients and *t* values of 7 factors (Table 5) showed that X_1 , X_2 , X_4 , and X_5 had positive effects on cordycepin production. X_6 had negative effects. The variable affects with a confidence level above 95% are considered as significant factors. Based on these results, three factors (X_1 , sucrose; X_4 , $K_2HPO_4 \cdot 3H_2O$;

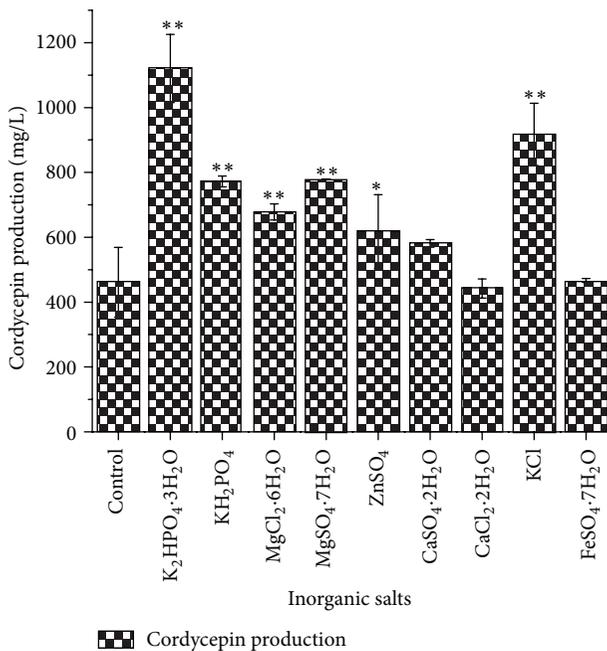


(a)

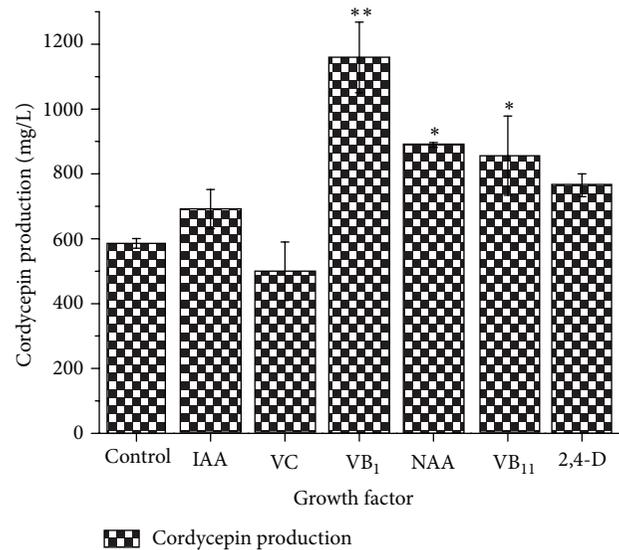


(b)

FIGURE 2: Effects of carbon sources and nitrogen sources on the production of cordycepin: carbon sources (a); nitrogen sources (b); *5% significance level (test group versus control group); **1% significance level (test group versus control group); #5% significance level (control group versus test group).



(a)



(b)

FIGURE 3: Effects of inorganic salt and growth factors on the production of cordycepin: inorganic salt (a); growth factors (b); *5% significance level (test group versus control group); **1% significance level (test group versus control group).

TABLE 5: Results of regression analysis of Plackett-Burman design.

Symbol	Effect	Coefficient	Regression analysis		
			Standard error	T	P
		845.82	32.86	25.74	0.000**
X_1	190.88	95.44	32.86	2.90	0.027*
X_2	149.23	74.62	32.86	2.27	0.064
X_3	-40.85	-20.42	32.86	-0.62	0.557
X_4	244.10	122.05	32.86	3.71	0.010*
X_5	62.81	31.41	32.86	0.96	0.376
X_6	-176.15	-88.08	32.86	-2.68	0.037*
X_7	-127.39	-63.69	32.86	-1.94	0.101
Ct Pt		276.82	73.48	3.77	0.009**

* 5% significance level; ** 1% significance level; X_1 - X_7 are symbols shown in Table 1.

TABLE 6: Experimental design and responses of the central composite design for carbon sources and inorganic salts.

Run	Variables Code			Y (mg/L) Cordycepin production	Run	Variables Code			Y (mg/L) Cordycepin production
	X_1	X_4	X_6			X_1	X_4	X_6	
1	-1	-1	-1	1080.55 ± 109.69	11	0	0	0	1399.43 ± 124.44
2	1	-1	-1	1359.48 ± 12.61	12	0	0	0	1415.43 ± 42.13
3	-1	1	-1	1158.59 ± 12.15	13	0	0	0	1487.42 ± 16.38
4	1	1	-1	1289.90 ± 47.00	14	0	0	0	1409.43 ± 155.22
5	-1	-1	1	980.55 ± 34.72	15	-1.6818	0	0	876.91 ± 16.69
6	1	-1	1	1097.48 ± 52.42	16	1.6818	0	0	1290.00 ± 14.71
7	-1	1	1	987.95 ± 2.89	17	0	-1.6818	0	1110.57 ± 157.63
8	1	1	1	1117.38 ± 116.84	18	0	1.6818	0	1344.66 ± 65.54
9	0	0	0	1485.38 ± 12.19	19	0	0	-1.6818	958.39 ± 224.14
10	0	0	0	1333.48 ± 94.22	20	0	0	1.6818	958.00 ± 75.82

and X_6 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) were considered as significant for cordycepin production by static liquid culture methodology.

3.5. Optimization by Response Surface Methodology for Carbon Sources and Inorganic Salts. In order to evaluate the influence of medium component on cordycepin production, sucrose, $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ should be examined. The levels of variables for central composite design experiments were selected according to the above results of Plackett-Burman design. Table 6 shows the detailed experimental design and results. Regression analysis was performed to fit the response function (cordycepin production) with the experimental data. From the variables obtained (Table 6), the model is expressed by (1), which represents cordycepin production (Y_1) as a function of sucrose (X_1), $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ (X_4), and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (X_6) concentrations:

$$\begin{aligned}
 Y_1 = & 1419.68 - 98.95X_1 + 31.45X_4 - 51.68X_6 \\
 & - 16.89X_1X_4 - 20.48X_1X_6 + 2.36X_4X_6 \\
 & - 106.03X_1^2 - 55.06X_4^2 - 150.31X_6^2.
 \end{aligned} \quad (1)$$

Results of F -test analysis of variance (ANOVA) showed that the regression was statistically significant at 95% and 99% confidence levels (Table 7). The " F value" of the model was 9.21, and the value of " $\text{Prob} > F$ " < 0.01 indicated that the

model was significant. In this case, linear terms of X_1 and quadratic terms of X_1^2 , X_4^2 , X_6^2 were significant of model terms for cordycepin production. The " $Lack\ of\ Fit\ F\ value$ " of 0.0903 implied that the " $Lack\ of\ Fit$ " was not significant relative to the pure error ($P > 0.05$). The $\text{Pred-}R^2$ of 0.3183 was not as close to the $\text{Adj-}R^2$ of 0.7954 as one might normally expect. The result suggested that some factors were not considered in the model. However, the "Adeq Precision" of 8.173 indicated that the model was adequate for prediction production of cordycepin.

The response surface plot obtained from (1) is shown in Figure 4. It is evident that cordycepin production reached its maximum at a combination of coded level (X_1 , sucrose, level 0.47; X_4 , $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, level 0.21; X_6 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, level -0.20) when using canonical analysis of the Design-Expert Version 8.0.5b software package. The model predicted a maximum response of 1451.43 mg/L cordycepin production at levels of sucrose 24.7 g/L, $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ 1.11 g/L, and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.90 g/L as optimized medium components.

3.6. Effects of Nucleoside Analogue and Amino Acid on the Production of Cordycepin. Chassy and Suhadolnik [43] reported that adenine and adenosine were precursors for cordycepin synthesis. Amino acids were regarded as the best substance for improved cordycepin production [4, 23]. Based on these results, among 10 different kinds of nucleoside

TABLE 7: ANOVA for response surface quadratic polynomial model for carbon sources and inorganic salts.

Source	Sum of quares	df	Mean Square	F-value	P-value Prob > F
Model	6.507E + 005	9	72300.77	9.21	0.0009**
X ₁ -X ₁	1.337E + 005	1	1.337E + 005	17.03	0.0021**
X ₄ -X ₄	13504.30	1	13504.30	1.72	0.2190
X ₆ -X ₆	36477.48	1	36477.48	4.65	0.0565
X ₁ X ₄	2282.55	1	2282.55	0.29	0.6016
X ₁ X ₆	3356.89	1	3356.89	0.43	0.5279
X ₄ X ₆	44.38	1	44.38	5.653E - 003	0.9416
X ₁ ²	1.620E + 005	1	1.620E + 005	20.63	0.0011**
X ₄ ²	43685.18	1	43685.18	5.56	0.0400**
X ₆ ²	3.256E + 005	1	3.256E + 005	41.47	<0.0001**
Residual	78513.73	10	7851.37		
Lack of Fit	61670.32	5	12334.06	3.66	0.0903
Pure Error	16843.41	5	3368.68		
Cor Total	7.292E + 005	19			

R² = 0.8923; CV = 7.34%; Pred-R² = 0.3183; Adj-R² = 0.7954; Adeq Precision = 8.173; *5% significance level; **1% significance level.

TABLE 8: Experimental design and responses of the central composite design for amino acid, nucleoside analogue, and time.

Run	Variables Code			Y (mg/L) Cordycepin production	Run	Variables Code			Y (mg/L) Cordycepin production
	A	B	C			A	B	C	
1	-1	-1	-1	1383.01 ± 41.53	11	0	0	0	2041.25 ± 54.70
2	1	-1	-1	1422.52 ± 39.41	12	0	0	0	2020.97 ± 73.70
3	-1	1	-1	1216.88 ± 8.69	13	0	0	0	1998.18 ± 49.48
4	1	1	-1	1857.51 ± 164.86	14	0	0	0	2068.60 ± 72.79
5	-1	-1	1	1216.87 ± 253.38	15	-1.6818	0	0	1590.14 ± 222.14
6	1	-1	1	1111.18 ± 170.50	16	1.6818	0	0	1573.90 ± 776.16
7	-1	1	1	1536.05 ± 75.17	17	0	-1.6818	0	1636.44 ± 65.23
8	1	1	1	851.70 ± 17.01	18	0	1.6818	0	1527.98 ± 177.46
9	0	0	0	2073.27 ± 65.85	19	0	0	-1.6818	1211.14 ± 82.58
10	0	0	0	1743.09 ± 14.81	20	0	0	1.6818	676.97 ± 142.74

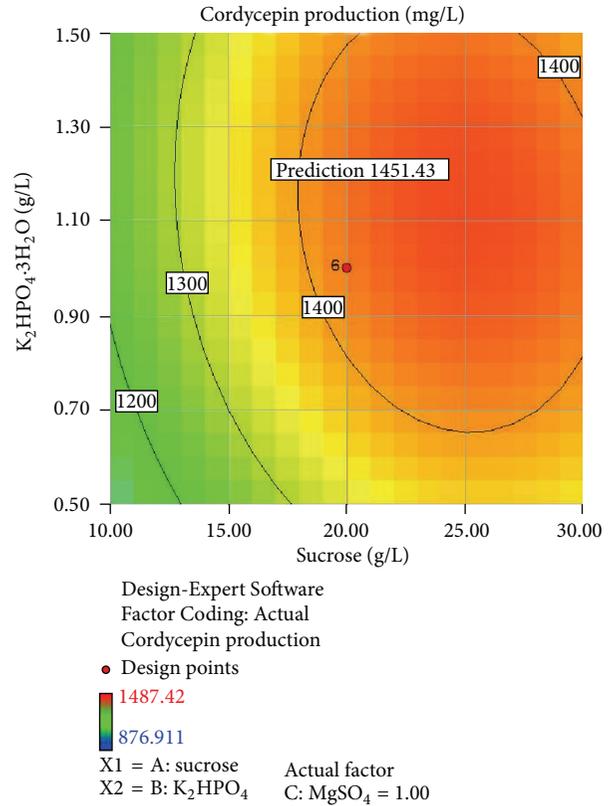
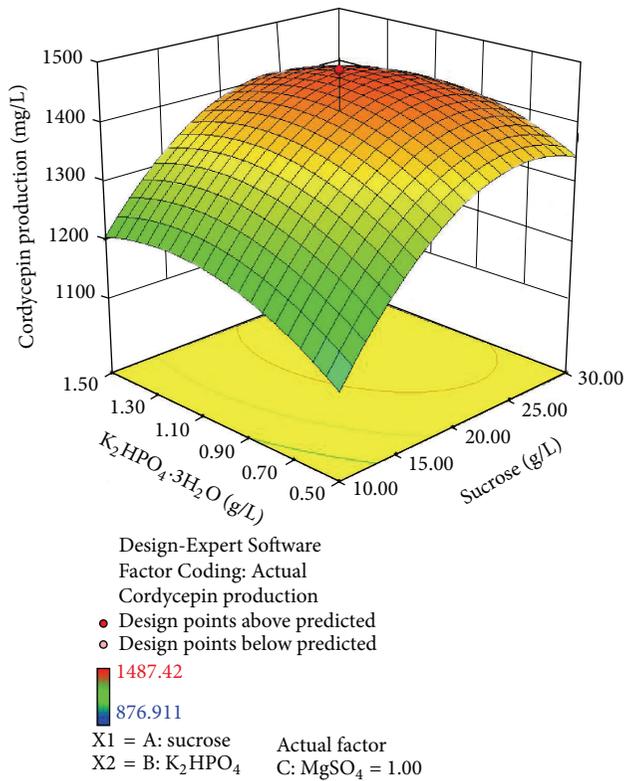
analogue were supplemented for 1 g/L in this study. As shown in Figure 5(a), cordycepin production increased obviously in the medium with hypoxanthine, thymine, and thymidine additives. The highest production of cordycepin was achieved, when hypoxanthine was used as the nucleoside analogue. Hypoxanthine’s molecular structure is similar to purine bases found in cordycepin. Substituent on purine bases structure is -OH on hypoxanthine rather than -NH₂. The -OH should be replaced in metabolic pathways. In addition, among 14 different amino acids were tested for 8 g/L. As shown in Figure 5(b), L-alanine can improve cordycepin production. Previous research showed that adenine, adenosine, and glycine were good additives for increased cordycepin production [4, 23, 26, 27]. L-alanine may be an important nutritional element for *C. militaris* or component of cordycepin production. Hypoxanthine and L-alanine were the best additives to promote cordycepin production in this study.

3.7. Optimization by Response Surface Methodology for Amino Acid, Nucleoside Analogue, and Fermentation Time. Similarly, central composite design was also applied to study

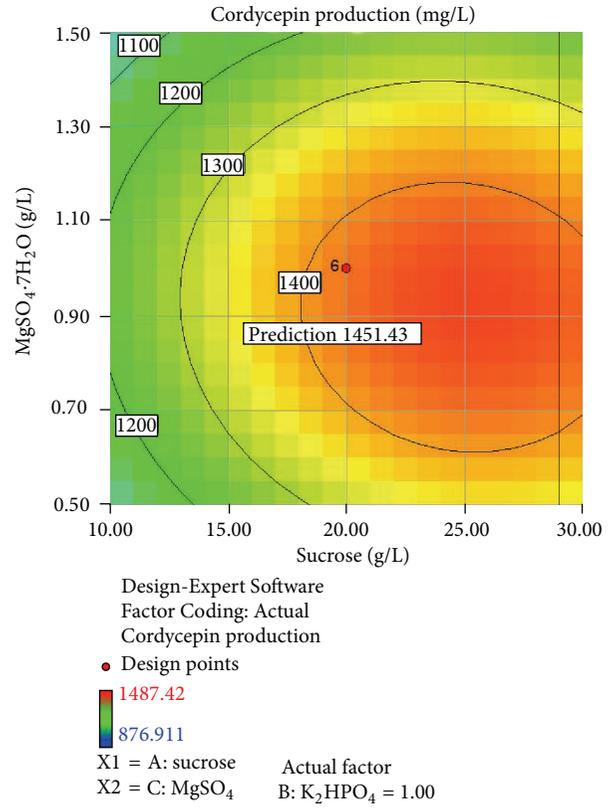
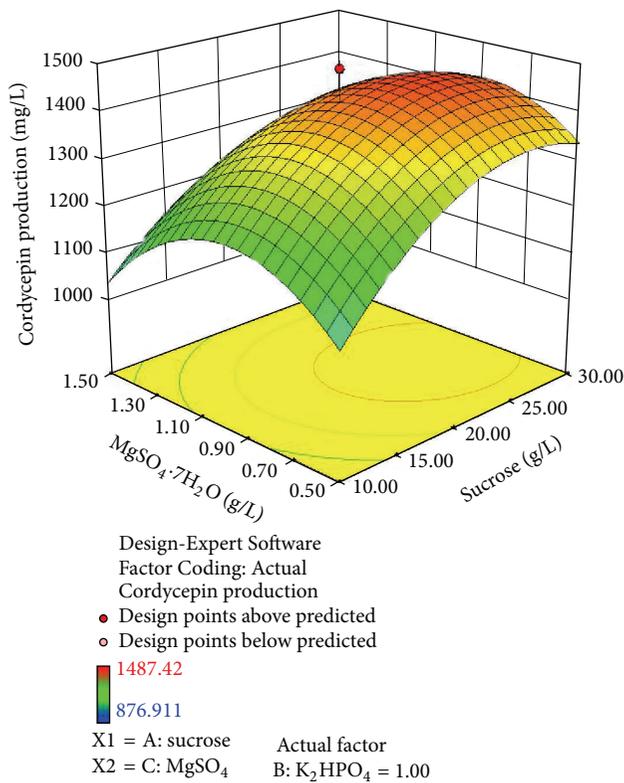
the significant factors (hypoxanthine, L-alanine, and culture time) and their optimal levels. Figure 6 shows the morphological characteristics of *C. militaris* in 1000 mL glass jars after fermentation by static liquid fermentation. Table 8 shows the detailed experimental design and results. Regression analysis was performed to fit the response function (cordycepin production) with the experimental data. From the variables obtained (Table 9), the model was expressed by (2), which represented cordycepin production (Y₂) as a function of hypoxanthine (A), L-alanine (B), and culture time (C, time), concentrations:

$$\begin{aligned}
 Y_2 = & 1991.13 - 10.05A + 10.70B - 151.02C \\
 & + 2.81AB - 183.77AC - 26.14BC \\
 & - 146.09A^2 - 146.03B^2 - 371.65C^2.
 \end{aligned}
 \tag{2}$$

Results of F-test analysis of variance (ANOVA) showed that the regression was statistically significant at 95% and 99% confidence level (Table 9). The “F value” of the model was 11.91, and the value of “Prob > F” < 0.01 indicated that the model was significant. In this case, linear terms



(a)



(b)

FIGURE 4: Continued.

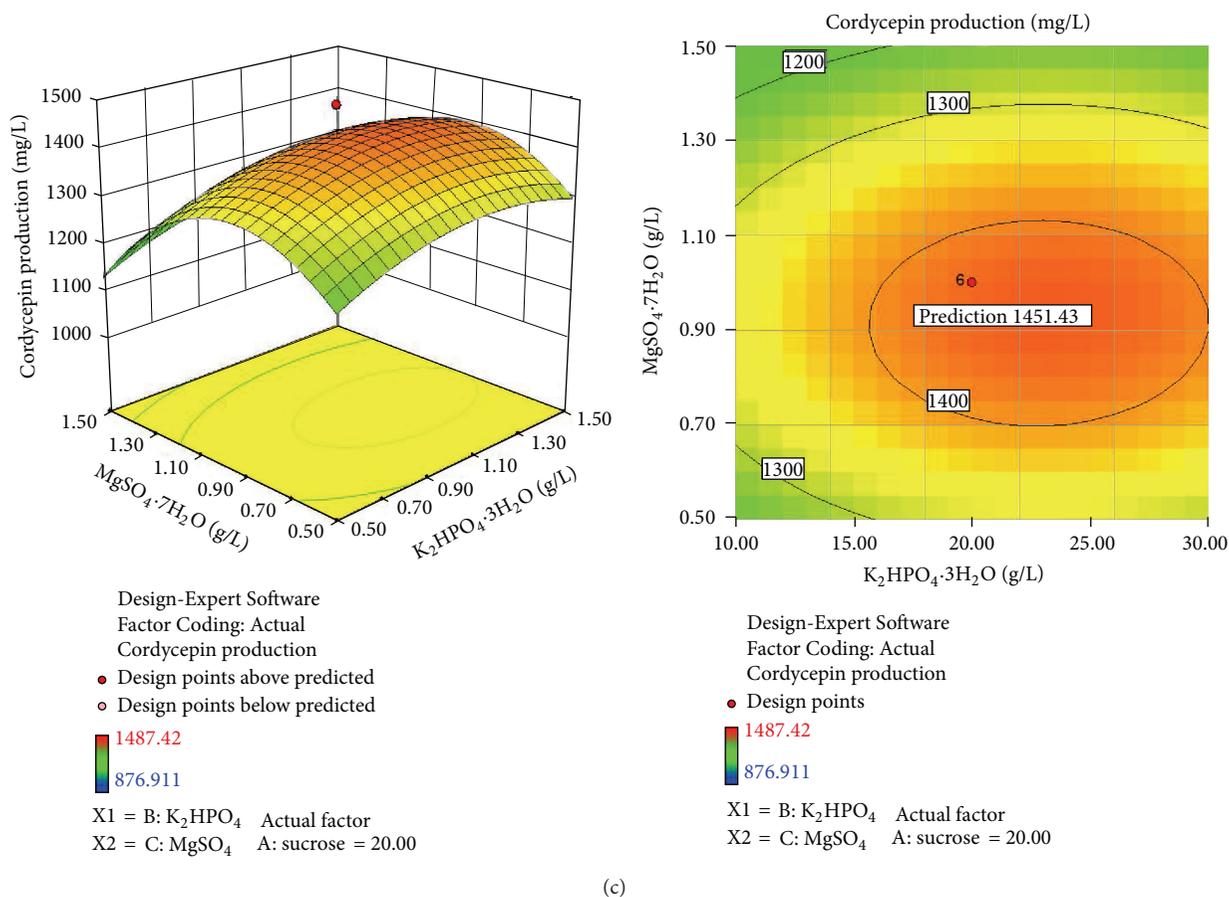


FIGURE 4: Three-dimensional response surface plots and two-dimensional contour plots for cordycepin production by *C. militaris* (strain CGMCC2459) showing variable interactions of (a) sucrose and K₂HPO₄·3H₂O; (b) sucrose and MgSO₄·7H₂O; (c) K₂HPO₄·3H₂O and MgSO₄·7H₂O.

TABLE 9: ANOVA for response surface quadratic polynomial model for amino acid, nucleoside analogue, and time.

Source	Sum of squares	df	Mean Square	F-value	P-value Prob > F
Model	2.899E + 006	9	3.221E + 005	11.91	0.0003**
A-A	1378.39	1	1378.39	0.051	0.8260
B-B	1564.06	1	1564.06	0.058	0.8148
C-C	3.115E + 005	1	3.115E + 005	11.51	0.0068*
AB	63.06	1	63.06	2.331E - 003	0.9624
AC	2.702E + 005	1	2.702E + 005	9.99	0.0102*
BC	5468.49	1	5468.49	0.20	0.6626
A ²	3.076E + 005	1	3.076E + 005	11.37	0.0071**
B ²	3.073E + 005	1	3.073E + 005	11.36	0.0071**
C ²	1.990E + 006	1	1.990E + 006	73.58	<0.0001**
Residual	2.705E + 005	10	27053.63		
Lack of Fit	1.928E + 005	5	38561.98	2.48	0.1707
Pure Error	77726.41	5	15545.28		
Cor Total	3.169E + 006	19			

R² = 0.9146; CV = 10.70%; Pred-R² = 0.4162; Adj-R² = 0.8378; Adeq Precision = 11.222; *5% significance level; **1% significance level.

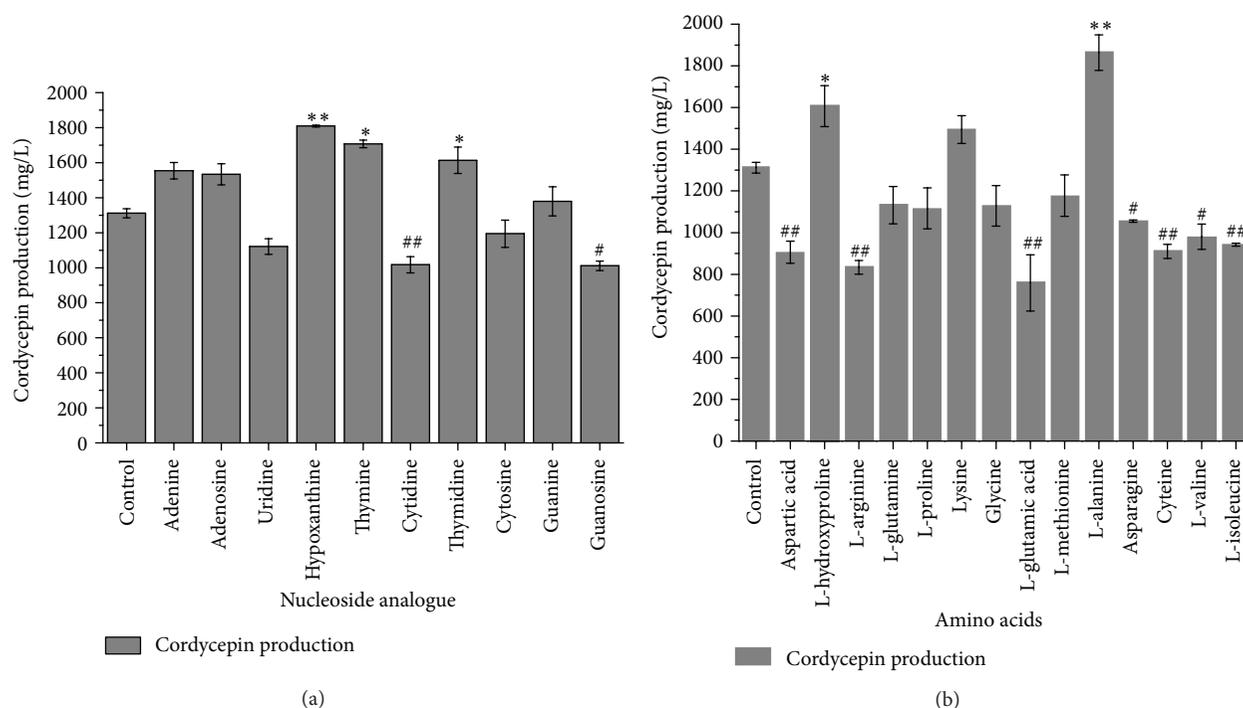


FIGURE 5: Effects of nucleoside analogue and amino acid on the production of cordycepin: nucleoside analogue (a); amino acid (b); *5% significance level (test group versus control group); **1% significance level (test group versus control group); #5% significance level (control group versus test group); ##1% significance level (control group versus test group).

of C ; interactive terms of AC ; and quadratic terms of A^2 , B^2 , and C^2 were significant in model terms for cordycepin production. The “Lack of Fit F value” of 0.1707 implied that the “Lack of Fit” was not significant relative to the pure error ($P > 0.05$). The $\text{Pred-}R^2$ of 0.4162 was not as close to the $\text{Adj-}R^2$ of 0.8378 as one might normally expect. The result suggested that some factors were not also considered in the model. However, the “Adeq Precision” of 11.222 indicated that the model was adequate for prediction production of cordycepin.

The response surface plot obtained from (2) is shown in Figure 7. It is evident that cordycepin production reached its maximum with a combination of coded level (A , hypoxanthine, level 0.11; B , L-alanine, level 0.06; C , time, level -0.23) by canonical analysis of the Design-Expert Version 8.0.5b software package. The model predicted a maximum response of 2008.48 mg/L cordycepin production at levels of hypoxanthine 5.45 g/L, L-alanine 12.23 g/L, and time 8.6 days (in the practical test 8 days) as optimized medium components.

In previous work, the orthogonal design method [44–46], Box-Behnken design [34, 47], and central composite design [31] were used to optimize culture conditions for cordycepin production by *Cordyceps* sp. These experimental designs have been successfully used to optimize medium for the mycelial growth and microbial metabolite production in liquid culture processes. In this study, static liquid culture conditions are optimized for the cordycepin production using response surface methodology and are an effective way to

enhance the productivity of cordycepin and biomass in *C. militaris*.

3.8. Verification Experiments and Batch Culture. Based on the results of response surface methodology, the optimized medium was prepared as follows: peptone 20 g/L; sucrose 24.7 g/L; $K_2HPO_4 \cdot 3H_2O$ 1.11 g/L; $MgSO_4 \cdot 7H_2O$ 0.90 g/L; VB_1 10 mg/L; hypoxanthine 5.45 g/L; and L-alanine 12.23 g/L. Five experiments were performed to confirm the above optimal culture requirements. The data were 2011.15 mg/L, 2000.69 mg/L, 1989.22 mg/L, 1969.6 mg/L, and 2061.37 mg/L, respectively. The average cordycepin production was 2006.41 ± 34.37 mg/L. The experimental values were particularly close to the predicted values (2008.48 mg/L). The result confirmed the model suited the predictive of hyperproduction of cordycepin by *C. militaris* in static liquid culture. Batch culture was carried for cordycepin production under optimized culture conditions (Figure 8).

3.9. In Vitro Cordycepin Production Using Liquid Culture in Other Studies. The highest report for cordycepin production was 14300 mg/L by Masuda et al. [29] (Table 10). In our experiment, cordycepin production at 2008.48 mg/L was lower. However, a maximum total content of cordycepin (1405.94 mg) was achieved in our study. This is a second higher report of cordycepin production in one single fermenter. The results showed that the culture conditions will provide an effective way for increasing cordycepin production.

TABLE 10: Cordycepin production in the medium by liquid culture in different studies.

No.	Methodology	Working volume of the medium v/v (mL/mL)	Cordycepin production (mg/L)	Total content of cordycepin in one bottle (mg)	References
1	Submerged culture	50/250	245.7	12.5	Mao et al., [32]
2	Submerged culture	50/250	420.5	21.03	Mao and Zhong [21]
3	Surface liquid culture	100/500	640	64	Masuda et al., [25]
4	Shaking + Static	100/250	2214.5	221.45	Shih et al., [34]
5	Surface liquid culture	100/500	2500	250	Masuda et al., [26]
6	Surface liquid culture	100/500	3100	310	Das et al., [30]
7	Surface liquid culture	100/500	8570	857	Das et al., [27]
8	Submerged culture	100/500	1644.21	164.42	Wen et al., [23]
9	Dark + Shaking	100/500	1015	101.5	Kang et al., [24]
10	Surface liquid culture	150/500	14300	2145	Masuda et al., [29]
11	Static liquid culture	700/1000	2008.48	1405.94	In this study

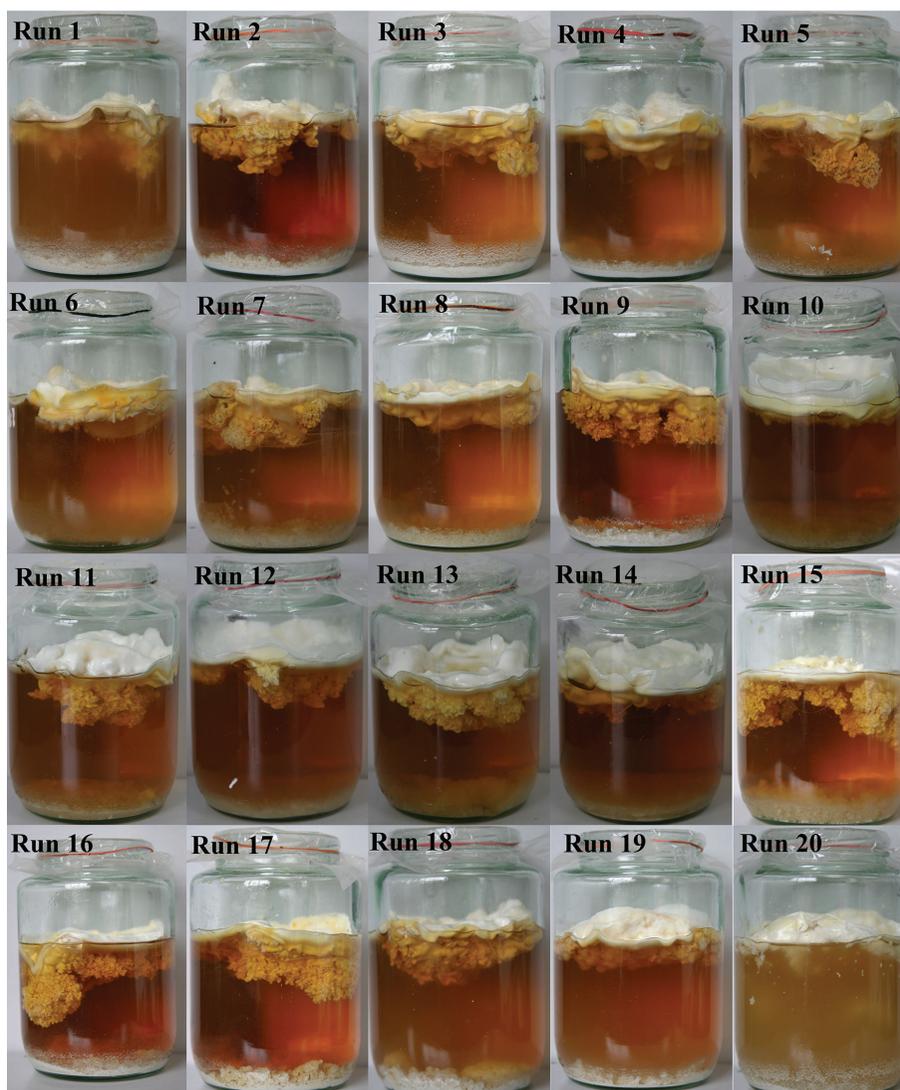
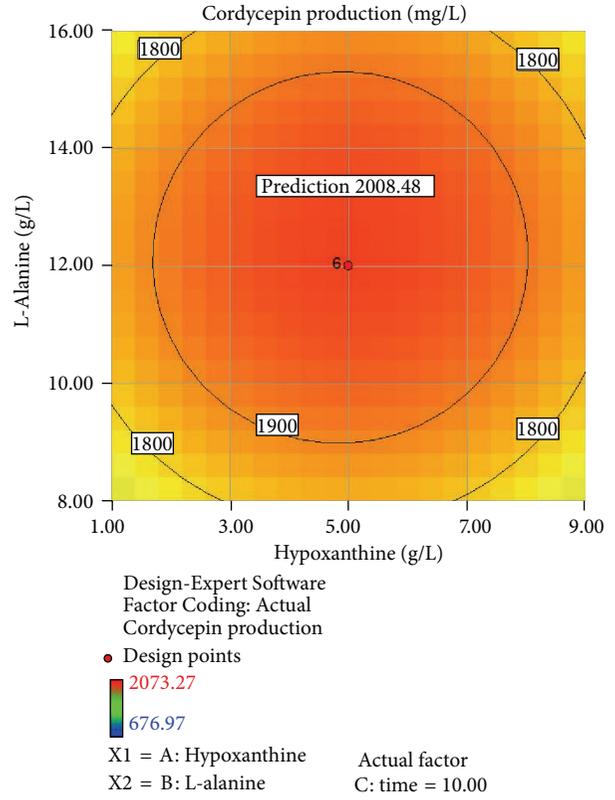
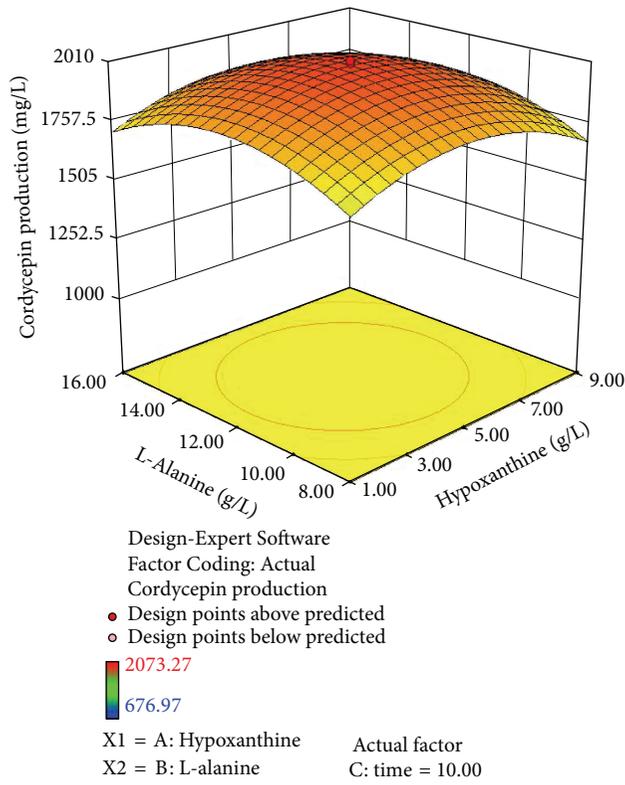
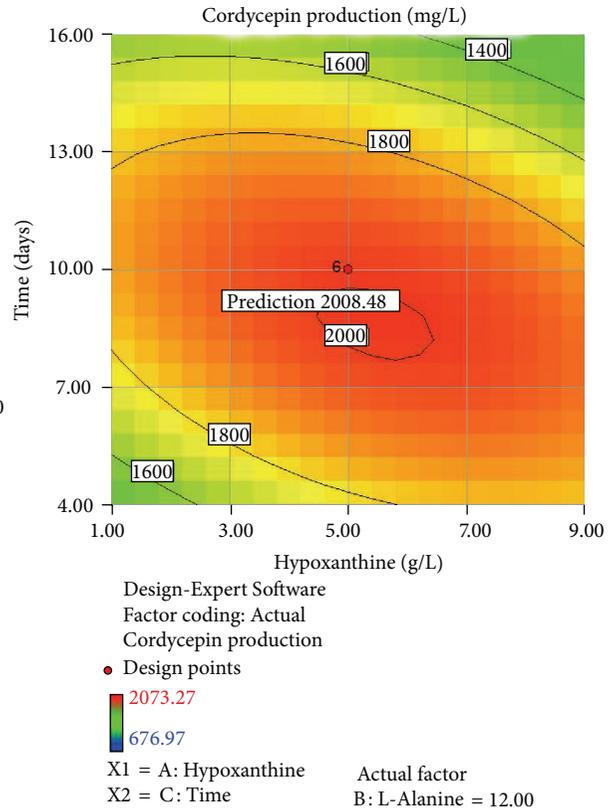
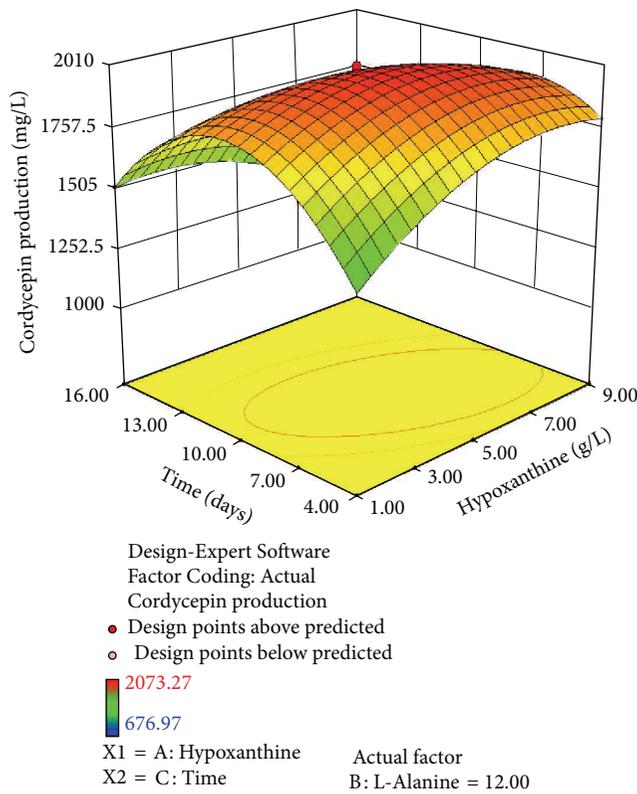


FIGURE 6: Morphology of *C. militaris* (strain CGMCC2459) in 700/1000 mL glass jars at the end of the fermentation process by response surface methodology: symbols in photos indicated 20 runs.



(a)



(b)

FIGURE 7: Continued.

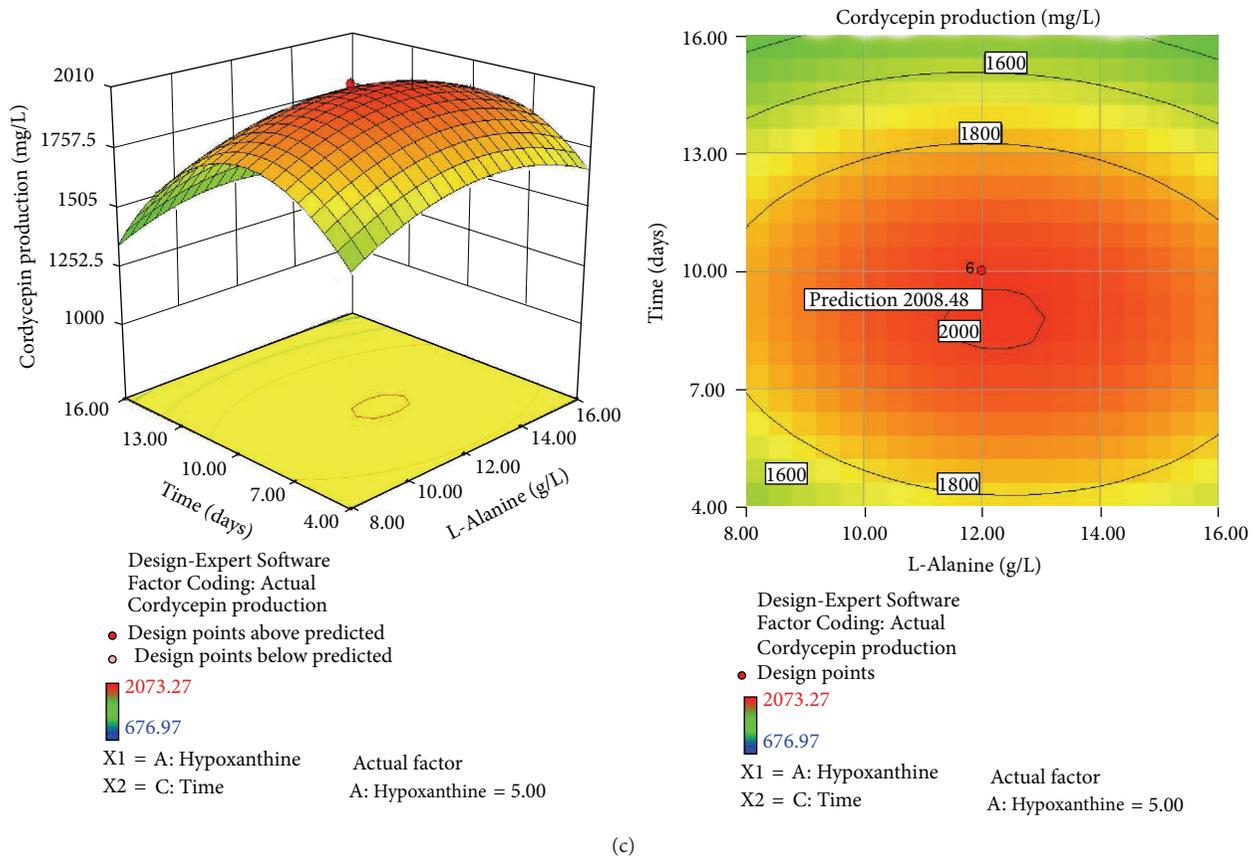


FIGURE 7: Three-dimensional response surface plots and two-dimensional contour plots for cordycepin production by *C. militaris* (strain CGMCC2459) showing variable interactions of (a) hypoxanthine and L-alanine; (b) hypoxanthine and time; (c) L-alanine and time.



FIGURE 8: Batch culture for cordycepin production under optimized culture conditions by static liquid culture using *C. militaris* (strain CGMCC2459).

4. Conclusion

In this work, single factor design, Plackett-Burman design, and central composite design were employed to establish the key factors and identify optimal culture conditions which improved cordycepin production by *C. militaris* CGMCC2459. Optimal media contained peptone 20 g/L; sucrose 24.7 g/L; $K_2HPO_4 \cdot 3H_2O$ 1.11 g/L; $MgSO_4 \cdot 7H_2O$

0.90 g/L; VB_1 10 mg/L; hypoxanthine 5.45 g/L; and L-alanine 12.23 g/L. Hypoxanthine and L-alanine were added to the optimal medium at 8.6 days. Optimal incubation conditions were 25°C at an unaltered pH of 35 days. Using these culture conditions, a maximum production of cordycepin was 2008.48 mg/L for 700 mL working volume in the 1000 mL glass jars, and total content of cordycepin reached 1405.94 mg/bottle (700 mL/1000 mL). This method provides

an effective way for increasing the cordycepin production at a large scale. The strategies used in this study could have a wide application in other fermentation process.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Chao Kang and Ting-Chi Wen contributed equally to this work.

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