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 Cordycepietaceae [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], antivirus [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; KH₂PO₄ 1 g/L; and MgSO₄·7H₂O 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; KH₂PO₄ 1 g/L; and MgSO₄·7H₂O 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, NH₄NO₃, NaNO₃, NH₄Cl, casein, and carbamide) and inorganic salts (MgCl₂·6H₂O, MgSO₄·7H₂O, KCl, ZnSO₄, CaCl₂·2H₂O, CaSO₄·2H₂O, FeSO₄·7H₂O, and K₂HPO₄·3H₂O) were also studied using static culture. Growth factors (Vitamin B₁ (VB₁), Vitamin B₂ (VB₂), Vitamin B₆ (VB₆), Vitamin B₁₂ (VB₁₂), 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 ×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 ×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 KH₂PO₄, 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 (X₁), peptone (X₂), K₂HPO₄·3H₂O (X₃), MgSO₄·7H₂O (X₄), and VB₁ (X₅) 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.
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$_2$HPO$_4$, 3H$_2$O, and MgSO$_4$, 7H$_2$O) 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 ± 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.

#### 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 ± 56.72 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.

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

| Symbol | Variables              | Experimental value |
|--------|------------------------|--------------------|
| $X_1$  | Sucrose (g/L)          | Low ($-1$)          |
| $X_2$  | Peptone (g/L)          | Low ($-1$)          |
| $X_3$  | Virtual 1              | High ($+1$)         |
| $X_4$  | $K_2$HPO$_4$, 3H$_2$O (g/L) | Low ($-1$)          |
| $X_5$  | VB$_5$ (g/L)           | High ($+1$)         |
| $X_6$  | MgSO$_4$, 7H$_2$O (g/L) | Low ($-1$)          |
| $X_7$  | Virtual 2              | High ($+1$)         |

**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) × working volume (mL)).
One effect was negative since, in excess, nitrogen 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. In previous work, nitrogen showed a regulating role in cordycepin production and had two effects [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 1 g/L. Among the 8 nitrogen sources tested, peptone, yeast extract, beef extract, casein, and NH₄NO₃ 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.

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

| Runs | X₂ | X₃ | X₄ | X₅ | X₆ | X₇ |  Y (mg/L) Cordycepin production |
|------|----|----|----|----|----|----|---------------------------------|
| 1    | 1  | −1 | 1  | −1 | −1 | 1  | 812.36 ± 26.83                  |
| 2    | 1  | 1  | −1 | 1  | −1 | −1 | 1395.18 ± 8.4                   |
| 3    | −1 | 1  | 1  | −1 | 1  | −1 | 900.25 ± 10.29                  |
| 4    | 1  | −1 | 1  | 1  | −1 | −1 | 802.45 ± 45.43                  |
| 5    | 1  | 1  | −1 | 1  | 1  | −1 | 1097.66 ± 25.57                 |
| 6    | 1  | 1  | −1 | 1  | 1  | 1  | 845.87 ± 24.94                  |
| 7    | −1 | 1  | 1  | −1 | −1 | −1 | 786.35 ± 7.61                   |
| 8    | −1 | −1 | 1  | 1  | −1 | −1 | 805.08 ± 29.1                   |
| 9    | −1 | −1 | −1 | 1  | 1  | −1 | 920.48 ± 16.21                  |
| 10   | 1  | −1 | −1 | −1 | −1 | 1  | 694.01 ± 79.51                  |
| 11   | −1 | 1  | −1 | −1 | −1 | 1  | 497.28 ± 4.44                   |
| 12   | −1 | −1 | −1 | −1 | −1 | −1 | 592.83 ± 16.13                  |
| 13   | 0  | 0  | 0  | 0  | 0  | 0  | 1134.14 ± 2.59                  |
| 14   | 0  | 0  | 0  | 0  | 0  | 0  | 1100.21 ± 0.08                  |
| 15   | 0  | 0  | 0  | 0  | 0  | 0  | 1133.56 ± 1.85                  |

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

| Symbol | Variables | Code level |
|--------|-----------|------------|
| A      | Hypoxanthine (g/L) | −1.6818 −1 0 1 1.6818 |
| B      | L-alanine (g/L) | −1.6818 −1 0 1 1.6818 |
| C      | Culture time (days) | −0.09 4 10 16 20.09 |

The highest cordycepin production (1120.30 ± 105.28 mg/L) by *C. militaris* was observed in medium, when K₂HPO₄·3H₂O was used as an inorganic salt. KH₂PO₄, MgSO₄·7H₂O, KCl, and MgCl₂·6H₂O were also useful inorganic salts. At last, MgSO₄·7H₂O and K₂HPO₄·3H₂O 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 VB₁, NAA, and VB₁₁ (Figure 3(b)). Maximum cordycepin production (1159.34 ± 109.01 mg/L) occurred when VB₁ 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 X₁, X₂, X₄, and X₅ had positive effects on cordycepin production. X₆ had negative effects. The variable affects with a confidence level above 95% are considered as significant factors. Based on these results, three factors (X₁, sucrose; X₄, K₂HPO₄·3H₂O;
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).

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).
and $X_6$, MgSO$_4\cdot$7H$_2$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, $K_2$HPO$_4\cdot$3H$_2$O, and MgSO$_4\cdot$7H$_2$O should be examined. The levels of variables for central composite design experiments were selected according to the above results of Plackett-Burman design. Table 6 shows the detailed experimental design and results. Regression analysis was performed to fit the response function (cordycepin production) with the experimental data. From the variables obtained (Table 6), the model is expressed by (1), which represents cordycepin production ($Y_1$) as a function of sucrose ($X_1$), $K_2$HPO$_4\cdot$3H$_2$O ($X_4$), and MgSO$_4\cdot$7H$_2$O ($X_6$) concentrations:

$$Y_1 = 1419.68 - 98.95X_1 + 31.45X_4 - 51.68X_6$$

$$- 16.89X_1X_4 - 20.48X_1X_6 + 2.36X_4X_6$$

(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 “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 Pred-$R^2$ of 0.3183 was not as close to the 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; $X_4$, $K_2$HPO$_4\cdot$3H$_2$O, level 0.21; $X_6$, MgSO$_4\cdot$7H$_2$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, $K_2$HPO$_4\cdot$3H$_2$O 1.11 g/L, and MgSO$_4\cdot$7H$_2$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 squares | df | Mean Square | F-value | P-value | Prob > F |
|-------------|----------------|----|-------------|---------|---------|----------|
| Model       | 6.507E + 005   | 9  | 72300.77    | 9.21    | 0.0009**|          |
| X₁-X₁       | 1.337E + 005   | 1  | 1.337E + 005| 17.03   | 0.0021**|          |
| X₄-X₄       | 13504.30       | 1  | 13504.30    | 1.72    | 0.2190  |          |
| X₆-X₆       | 36477.48       | 1  | 36477.48    | 4.65    | 0.0565  |          |
| X₁-X₄       | 2282.55        | 1  | 2282.55     | 0.29    | 0.6016  |          |
| X₁-X₆       | 3356.89        | 1  | 3356.89     | 0.43    | 0.5279  |          |
| X₄-X₆       | 44.38          | 1  | 44.38       | 5.653E−003| 0.9416 |          |
| X₁²         | 1.620E + 005   | 1  | 1.620E + 005| 20.63   | 0.0011**|          |
| X₄²         | 43685.18       | 1  | 43685.18    | 5.56    | 0.0400**|          |
| X₆²         | 3.256E + 005   | 1  | 3.256E + 005| 41.47   | <0.0001**|          |
| Residual    | 78513.73       | 10 | 78513.73    |         |         |          |
| 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 |
|-----|----------------|-------------------------------|-----|----------------|-------------------------------|
| 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          | 2088.60 ± 72.79              |
| 5   | −1 −1 1        | 1216.87 ± 253.38             | 15  | −1.6818 0      | 1590.14 ± 222.14             |
| 6   | 1 −1 1         | 1111.18 ± 170.50             | 16  | 1.6818 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:

\[
Y₂ = 1991.13 - 10.05A + 10.70B - 151.02C + 2.81AB - 183.77AC - 26.14BC - 146.09A^2 - 146.03B^2 - 371.65C^2.
\]

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...
Design-Expert Software
Factor Coding: Actual
Cordycepin production
- Design points above predicted
- Design points below predicted

X1 = A: sucrose
X2 = B: K2HPO4
C: MgSO4 = 1.00

Figure 4: Continued.
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*          |
| A\(^2\)      | 63.06          | 1  | 63.06       | 2.331E - 003| 0.9624          |
| B\(^2\)      | 2.702E + 005   | 1  | 2.702E + 005| 9.99    | 0.0102*          |
| C\(^2\)      | 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 |             |         |                  |

\(R^2 = 0.9416; CV = 10.70%;\) Pred-\(R^2 = 0.4162;\) Adj-\(R^2 = 0.8378;\) Adeq Precision = 11.222; *5% significance level; **1% significance level.
of C; interactive terms of AC; and quadratic terms of A², B², and C² 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 Pred-R² of 0.4162 was not as close to the Adj-R² 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; K₂HPO₄·3H₂O 1.11 g/L; MgSO₄·7H₂O 0.90 g/L; VB₈, 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.
Design-Expert Software  
Factor Coding: Actual  
Cordycepin production  
- Design points above predicted  
- Design points below predicted

X1 = A: Hypoxanthine  
X2 = B: L-alanine  
C: time = 10.00

(a)

X1 = A: Hypoxanthine  
X2 = B: L-alanine  
C: time = 10.00

(b)

Figure 7: Continued.
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$_2$HPO$_4$·3H$_2$O 1.11 g/L; MgSO$_4$·7H$_2$O 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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