Production of cordycepin by *Cordyceps militaris* using submerged liquid culture: Optimization of the culture medium and repeated batch fermentation

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**Abstract**

Cordycepin (3'-deoxyadenosine) is one of the most versatile metabolites of *Cordyceps militaris* due to its broad spectrum of biological activity. In this study, response surface methodology (RSM) was applied to optimize the medium components for the cordycepin production by submerged liquid culture. The optimal parameters for cordycepin production within the experimental range of the variables researched was at 30.16 g/L glucose, 10.00 g/L yeast extract, 2.040 g/L KH₂PO₄, and 1.460 g/L MgSO₄. At this condition, the cordycepin was predicted to be 63.69 mg/L. The maximum cordycepin obtained experimentally was 64.15 mg/L, which confirmed the model prediction. The repeated batch operation was proved to be an efficient method to increase the cordycepin yield. During the repeated batch fermentation, the cordycepin yields and productivity of the 2nd to 6th cycles are higher than that of 1st cycle.

**Key words:** *Cordyceps militaris*, cordycepin, medium component, response surface design, repeated batch operation.

**Introduction**

*Cordyceps militaris*, known as one of the Chinese medicinal mushrooms, is an entomopathogenic fungus belonging to the class Ascomycetes, Hypocreales, Clavicipitaceae and *Cordyceps*¹. The fungus invades and proliferates within a specific insect, eventually killing the host. After passing the winter inside the host, it forms a fruiting body on the surface of the host insect’s cadaver in the next summer². *Cordyceps militaris* has received extensive attention for medical application due to its various physiological activities including cordycepin, cordyceps acid, cordyceps polysaccharide (CSPS), selenium³, ⁴. Cordycepin (3’-deoxyadenosine, an analogue of adenosine) is the major active constituent of *C. militaris* and was first reported as a metabolite that was isolated from a culture broth of *C. militaris*⁵. Cordycepin has been reported to have a large spectrum of biological activities, including the inhibition of inflammation⁶, induction of apoptosis⁷, inhibition of platelet aggregation⁸, inhibition of cell migration and invasiveness⁹, and inhibition of cell proliferation¹⁰. It also can reduce tumor formation in a model of metastasis in mice¹¹ and has therefore been proposed as a cancer drug. The low doses of cordycepin can reduce the length of poly (A) tails and the proliferation of NIH3T3 fibroblasts. The higher doses of the cordycepin inhibit the cell attachment and reduce focal adhesions. The cordycepin shut down the mTOR5 pathway, a signal transduction pathway that control proliferation, cell adhesion, and protein synthesis¹².

As the specificity of host and the particularity of growth environment, the natural resources of *C. militaris* are limited. The fruiting bodies of this fungus can be successfully obtained through artificial cultivation in insect pupa or other solid medium¹³. However, the fruiting bodies of wild *C. militaris* grow extremely slowly in insect pupa or other solid medium, which need about 30-45 days; moreover, their growth is restricted to a specific area and their sizes are very small¹³. Thus, the above characters limited the large-scale production by solid culture. Liquid fermentation, including surface liquid culture or the optimum combination of submerged and static cultures, has been proven to be favorable for the higher production of cordycepin by *C. militaris*¹⁴. By liquid culture, about 97% of the cordycepin synthesized by *C. militaris* was excreted into the culture medium and the chemical composition of the mycelia obtained by surface liquid culture and the natural *C. militaris* are almost the same¹⁵. In addition, the liquid culture period is relatively short and its culture process can be optimized to achieve a highly productive process. The concentrations of carbon and nitrogen sources were optimized to obtained higher cordycepin production by the surface liquid culture of *C. militaris*¹⁴, ¹⁶ and submerged cultivation¹⁷. In addition, the effects of some additives were preliminarily examined¹⁵, ¹⁸. In the liquid fermentation process of *C. militaris*, we found that the fungus usually grows in two ways: Loose mycelia clumps or pellets. Bai et al.¹⁹ demonstrated that pellet form exhibited higher L-lactic acid yield than in clump form by *Rhizopus oryzae*, and were beneficial for repeated cycle fermentation. The application of the repeated batch operation could increase cordycepin productivity and reduce the required labor, time and energy leading to cut in the operation costs.

The objectivity of this research was to search for a more efficient
culture system for *C. militaris* by investigation of medium components to achieve higher cordycepin productivity. The response surface methodology (RSM) was used to optimize the effects of medium components on the cordycepin yield by submerged liquid culture of *C. militaris*, including the concentration of glucose, yeast extract, KH$_2$PO$_4$ and MgSO$_4$. In addition, applying a repeated batch culture of *C. militaris* was performed to enhance the cordycepin yield.

**Materials and Methods**

*Microorganism and preparation of medium:* The strain of *Cordyceps militaris* was stored in our laboratory, and was used throughout this study.

The microorganism was maintained on potato dextrose agar (PDA) slants and subcultured every month. Slants were incubated at 25°C for 7 days and then stored at 4°C. Liquid seed culture medium included glucose, 30 g/L; yeast extract powder, 10 g/L; KH$_2$PO$_4$, 2.0 g/L; MgSO$_4$$\cdot$7H$_2$O, 1.5 g/L. Fermentation medium was the same as seed culture medium.

*Culture conditions:* The mycelia of *C. militaris* were transferred to the seed culture medium (liquid volume of 130/250 mL flask) by punching out 1-2 blocks about 6 mm diameter agar discs from PDA slant; the seed culture was incubated at 25°C on a rotary shaker incubator at 120 rpm for 5 days. Then the fermentation medium (liquid volume of 40/100 mL) was inoculated with the seed culture (10%, v/v) and incubated in the same condition for 5 days.

**Analytical methods:** The determination and measurement of cordycepin were performed following the description by Shih et al. An accurate amount of cordycepin (Sigma, USA) was dissolved in water, to give various concentrations for calibration. For the analysis of extracellular cordycepin by *C. militaris*, the resulting culture filtrate was centrifuged at 13,000g for 5 min and the supernatant was filtered through a 0.45 µm membrane (Millipore, USA). The cordycepin concentration in the filtrate was analyzed by HPLC (Waters, USA) equipped with a UV detector (Waters model 2996) and a reverse phase C18 column (Waters XTerra RP-18 (150×4.6 mm 5 µm)). The mobile phase consisted of methanol and 0.02 M monopotassium phosphate (15/85, v/v). The flow rate was 1.0 mL/min; the injection volume was 20 µL and the column temperature was 40°C. The chromatogram was monitored by the UV absorbance at 260 nm.

**Experimental design for response surface methodology:** Response surface analysis is an optimization method, which is the system response as a function of one or more factors. To obtain a high cordycepin productivity, the response surface methodology (RSM) and Box-Behnken design was used for optimizing the medium components, including the concentration of glucose, yeast extract, KH$_2$PO$_4$ and MgSO$_4$. To decide the boundary of different variables for the optimization with response surface analysis (RSA), the effects of independent variables to cordycepin yield were investigated, including glucose concentration, yeast extract concentration, KH$_2$PO$_4$ concentration and MgSO$_4$ concentration in the medium (data not shown). The selected levels range of different variables were: glucose concentration ($X_1$) from 25 to 35 g/L; yeast extract concentration ($X_2$) from 8 to 12 g/L; KH$_2$PO$_4$ concentration ($X_3$) from 1.5 to 2.5 g/L; MgSO$_4$ concentration ($X_4$) from 1.0 to 2.0 g/L (Table 1).

A second-order polynomial equation (Eq. (1)) was developed to study the effects of variables on the yield. The equation indicates the effect of variables in terms of linear, quadratic, and interaction terms.

\[
Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{i i} X_i^2 + \sum \sum \beta_{i j} X_i X_j
\]

where $Y$ is the predicted response (cordycepin yield, mg/L), $\beta_0$ is a constant coefficient, $\beta_i$ is the linear coefficient, $\beta_{i i}$ is the quadratic coefficient, and $X_i, X_j$ are the coded independent variables or factors. The analysis of variance (ANOVA) was performed to evaluate significance of the model and coefficients. The quality of fit of the second-order model equation was expressed by the coefficient of determination $R^2$, and its statistical significance was determined by an F-test. The computer software used was Design Expert (version 7.0) by Stat-Ease, Inc. (USA).

**Repeated batch operation:** Repeated batch fermentation was carried out in flask using the optimal medium. The 1st cycle lasted five days; then, each of the next cycles lasted 3 days. The culture broth was replaced with a fresh medium using a syringe every 3 days from the 2nd cycle.

**Results and Discussion**

*Fitting the model:* For a four-factor, three-level design, the experimental trials were given by a set of points at the midpoint of each edge of a multidimensional cube and three replication of center points, resulting in a total number of 27 experiments. The experiments were repeated three times, and the cordycepin yields were the average values. The RSA design and the results obtained are shown in Table 2.

By applying multiple regression analysis on the experimental data, the model with the p-value less than 0.01 was statistically significant (Table 3), which implied that the results were fitted with a second-order polynomial equation (Eq. (1)). The results of the regression analysis are shown in Table 4, and the second-order polynomial equation obtained for cordycepin production is shown in Eq. (2) (in terms of coded levels):

\[
Y=60.02+2.71X_1+5.73X_2-0.57X_3-5.23X_4-2.18X_1X_2-0.21X_1X_3-10.37X_4^2
\]

where $X_i$ is glucose concentration, $X_2$ is yeast extract concentration, $X_3$ is KH$_2$PO$_4$ concentration, and $X_4$ is MgSO$_4$ concentration. From Table 4 and Eq. (2), the linear term of yeast extract and MgSO$_4$ concentration, the interaction between yeast extract and MgSO$_4$ concentration, and the quadratic term of glucose, yeast extract and MgSO$_4$ concentration have significant

<table>
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<tr>
<th>Level</th>
<th>Glucose (g/L)</th>
<th>Yeast extract (g/L)</th>
<th>KH$_2$PO$_4$ (g/L)</th>
<th>MgSO$_4$ (g/L)</th>
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<td>-1</td>
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<td>10</td>
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<td>12</td>
<td>2.5</td>
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Table 1. Uncoded and coded levels of independent variables used in the RSM design.
Table 3. ANOVA for the fitted quadratic polynomial model.

<table>
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<tr>
<th>Source</th>
<th>Degree of freedom</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F Value</th>
<th>p-value</th>
<th>p-value Prob&gt;F</th>
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<td>0.0076</td>
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<tr>
<td>Lack of fit</td>
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<td>22.81</td>
<td>1.42</td>
<td>0.4822</td>
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<tr>
<td>Pure error</td>
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<td>32.09</td>
<td>16.04</td>
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<tr>
<td>Cor total</td>
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<td></td>
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</table>

R^2 = 83.46%, Adj. R^2 = 64.17%

Table 4. Significance test of regression coefficients.

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<tr>
<th>Term</th>
<th>Sum of Squares</th>
<th>df</th>
<th>Mean Square</th>
<th>F-value</th>
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<td>x_{14}</td>
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<td>573.65</td>
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Figure 1. Response surface plots showing the effects of different factors on cordycepin yield. (A): The glucose concentration (X1 g/L) and yeast extract concentration (X2 g/L); (B): The glucose concentration (X1 g/L) and KH2PO4 concentration (X3 g/L); (C): The glucose concentration (X1 g/L) and MgSO4 concentration (X4 g/L); (D): The yeast extract concentration (X2 g/L) and KH2PO4 concentration (X3 g/L); (E): The yeast extract concentration (X2 g/L) and MgSO4 concentration (X4 g/L); (F): The KH2PO4 concentration (X3 g/L) and MgSO4 concentration (X4 g/L). Y: Cordycepin (mg/L).

With the p-value being 0.4822, which indicated that the above models were adequate to predict the cordycepin production within the range of variables studied. Thus, based on the above model, the optimum combination was found to be following: 30.16 g/L glucose, 10.00 g/L yeast extract, 2.040 g/L KH2PO4, and 1.460 g/L MgSO4. Under this condition, the yield of cordycepin predicted was 63.69 mg/L.

Verification of the calculated maximum was done with experiments that were performed in the culture media representing the optimum combination found, and the cordycepin yield of 64.15 mg/L (average of three repeats) was obtained, which confirmed with the model prediction (63.69 mg/L).

Analysis of response surface: The best way to visualize the influence of the independent variables on the dependent one is to draw surface response plots of the model. Fig. 1 shows the 3D surface interaction of glucose concentration, yeast extract concentration, KH2PO4 concentration and MgSO4 concentration on the cordycepin yield.

As shown in Fig. 1(A), it was evident that cordycepin increased with the elevation of yeast extract, when glucose concentration, KH2PO4 concentration and MgSO4 concentration were kept invariant. On the other hand, cordycepin increased effect on the cordycepin yield. After the neglect of insignificant terms (on the basis of p<0.05) (Table 4), the model Eq. (2) was modified to reduced fitted model Eq. (3):

Y = 60.02 + 5.73x_2 - 5.23x_4 + 3.20x_2x_4 - 8.66x_2^2 - 1.29x_4^2

(3)

From Table 3, the “lack of fit” of this model was insignificant.
gradually at begin and then decreased with the increase of glucose concentration, and the turning point was 30 g/L.

The effects of glucose concentration and KH$_2$PO$_4$ concentration on the cordycepin yield at the fixed 10 g/L yeast extract and 1.5 g/L MgSO$_4$ is shown in Fig. 1(B). It was observed that the cordycepin yield significantly increased with increasing glucose concentration at low glucose concentration and KH$_2$PO$_4$ concentration, when the glucose concentration above than 30 g/L, the cordycepin yield slightly decreased with the increase the glucose. At the given glucose concentration, the cordycepin yield changed slightly with the variation of KH$_2$PO$_4$ concentration.

The effects of glucose concentration and MgSO$_4$ concentration on the cordycepin yield is shown in Fig. 1(C). At the given glucose concentration, the cordycepin yield increased with the increasing of MgSO$_4$ concentration under the regions of 1.0-1.5 g/L; while above 1.5 g/L, the cordycepin yield decreased dramatically with the increasing of MgSO$_4$ concentration.

Fig. 1(D) shows the effect of yeast extract concentration and KH$_2$PO$_4$ concentration on the cordycepin yield at the fixed 30 g/L glucose and 1.5 g/L MgSO$_4$. It was evident that the yield increased slightly with the elevation of yeast extract concentration, when KH$_2$PO$_4$ concentration was kept invariant. The yield enhancement gradually began and then decreased with the increase of KH$_2$PO$_4$, and the turning point was 2.0 g/L. Therefore, 2.0 g/L KH$_2$PO$_4$ is the optimal condition.

As presented in Fig. 1(E), an increase in cordycepin yield was observed with the increase of yeast extract concentration when MgSO$_4$ concentration was fixed, and the augment tendency became slower with the addition of yeast extract concentration. When yeast extract concentration was kept invariant, the yield rose firstly and then decreased with MgSO$_4$ increasing, and the scope of optimum MgSO$_4$ concentration for the formation of cordycepin was at about 1.5 g/L.

Fig. 1(F) reveals that the interaction of the KH$_2$PO$_4$ concentration with MgSO$_4$ concentration to cordycepin production showed similar trend to that of glucose concentration and KH$_2$PO$_4$ concentration (Fig. 1(B)). Among these two factors, the MgSO$_4$ concentration showed dominant role to cordycepin production. Based on an overall consideration of various factors, the optimum KH$_2$PO$_4$ concentration with MgSO$_4$ concentration were about 2.0 g/L and 1.5 g/L.

Repeated batch operation: The mycelia morphology affects heat transfer, dissolved oxygen, mass transfer and other important parameters of the fermentation process; therefore a good morphology is the basis of the success of fermentation$^{21}$. During the liquid fermentation of C. militaris, the culture medium was filled with uniform pellets formed in the optimum medium and the diameter was about 2-3 mm (Fig. 2). Then the uniform pellets were used for repeated batch cultures by replacing with fresh medium and leaving the pellets in the bottle.

Fig. 3 shows the cordycepin yield of 6 cycles (20 days) of repeated batch fermentation. The cordycepin yield of the 1st cycle reached 63.91 mg/L, and the productivity was 9.306 mg/d. In repeated cycle fermentation, the cordycepin yield of 2nd and 3rd cycles kept a relative stable levels, 90.71 mg/L and 89.95 mg/L, respectively. From 4th to 6th cycles, the cordycepin yields decreased from 82.53 mg/L to 64.37 mg/L; and the productivity decreased from 17.29 mg/(Ld) to 10.7 mg/(Ld). The cordycepin yield and productivity of the 2nd to 6th cycles are higher than that of 1st cycle.

The above results demonstrated that the applications of the repeated batch is an efficient method to enhance the cordycepin yield by C. militaris using submerged liquid culture, which is identical to the results of the repeated batch of C. militaris by surface culture.$^{15}$

Conclusions

In this study, optimization of the submerged liquid culture medium components of C. militaris for cordycepin production was carried out. According to the response surface experimental design and analysis, the optimal medium components for cordycepin yield was at 30.16 g/L glucose, 10.00 g/L yeast extract, 2.040 g/L KH$_2$PO$_4$, and 1.460 g/L MgSO$_4$. At this condition, the cordycepin yield was predicted to be 63.69 mg/L. To obtaining higher cordecepin, the repeated batch operation was tested. The repeated batch operation can increase the cordycepin yields dramatically. Both the cordycepin yields and productivity of the 2nd to 6th cycles are higher than that of 1st cycle.

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