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# Optimization of Submerged Culture Conditions for the Mycelial Biomass and Bioactive Metabolites Production by *Cordyceps militaris*

**Yan Liu, Wei-Nan Yuan and Xuan-Wei Zhou***

Plant Biotechnology Research Center, Key Laboratory of Urban Agriculture (South) Ministry of Agriculture, School of Agriculture and Biology, Shanghai Jiaotong University, Shanghai - 200240, China.

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**This paper is concerned with optimization of submerged culture conditions for mycelial growth and bioactivity components production by *Cordyceps militaris*. Based on investigation of the most suitable carbon source and nitrogen source by one-factor-at-a-time method, the combination of carbon and nitrogen sources were optimized to investigate the effects on mycelial growth and bioactive components production using $L_9(3^4)$ orthogonal array. The effects of medium components on mycelial growth of *C. militaris* and adenosine production were in the order of soluble starch> dried silkworm chrysalis meal> fructose> soybean meal, and those on cordycepin production were in the order of fructose> soybean meal> dried silkworm chrysalis meal> soluble starch. The optimal medium combination, soluble starch 22.50 g/L, fructose 17.50 g/L, soybean meal 5.00 g/L and silkworm chrysalis meal 3.75 g/L, was beneficial for the accumulation of mycelial biomass (≥2.36 g/L), while the optimal medium combination included soluble starch 12.50 g/L, fructose 22.50 g/L, soybean meal 3.75 g/L and silkworm chrysalis meal 3.75 g/L for the accumulation of adenosine (≥193.67 μg/g), and soluble starch 12.50 g/L, fructose 17.50 g/L, soybean meal 3.75 g/L and silkworm chrysalis meal 5.00 g/L for the accumulation of cordycepin (≥243.33 μg/g).**

**Keywords:** *Cordyceps militaris*; Cordycepin; Adenosine; Mycelial biomass; $L_9(3^4)$orthogonal array.

*Cordyceps militaris* belongs to *Cordyceps, Clavicipitaceae, Hypocreales, Ascomycota*, which is the same genus with traditional Chinese medicine *Ophiocordyceps sinensis*. Usually it parasites lepidopterous larvae and forms fruiting body when the host pupates. Modern biochemistry and pharmacology studies have shown that, it contains many active components, such as cordycepin, cordycepic acid, polysaccharide, etc.[1,2], and has a comprehensive range of benefits, such as antibacterial effect, anti-tumor effect, enhancing immune function, preventing cardiovascular disease, benefiting liver and kidney, enriching consumptive disease, reducing phlegm, stopping bleeding, anti-aging, alleviating sleepless, etc.[3,4,5,6]. Compared with natural *O. sinensis*, *C. militaris* is similar in active ingredients, pharmacological functions and clinical effects. The contents of *C. militaris*'s essential nutrients and biologically active ingredients, such as cordycepin, cordycepic acid and polysaccharide, are the same with or even exceed *O. sinensis*[7,8]. Due to the strict requirements of growing environment and over-excavation, *O. sinensis* is in short supply. Since *C. militaris* is more likely to grow successfully than *O. sinensis* in artificial cultivation modes, the study of *C. militaris* has aroused great interest of researchers all over the world. Researchers hope to cultivate *C. militaris*

* To whom all correspondence should be addressed.
Tel.: +86-21-34205778; Fax: +86-21-65642425;
E-mail: xuanweizhou@sjtu.edu.cn

artificially, replacing *O. sinensis*, and to compensate for the shortage of natural *O. sinensis*. In that case, much more consumers will be able to enjoy the benefits of *O. sinensis*[9].

As with other edible fungi, the artificial cultivation of *C. militaris* includes solid cultivation and submerged fermentation. Solid cultivation includes three stages, mycelial growth, differentiation and growth of fruiting body, which is similar to the development process of natural *C.militaris*[10,11]. But this cultivation method isn't suitable for large-scale factory production because of the long cultivation cycle and high cost of production. Submerged fermentation refers to the processes that the *Cordyceps* fungus was inoculated in liquid medium which contains all the nutrients needed for the growth of the fungus *Cordyceps* mycelium and cultured under suitable conditions to form mycelium breeding. Submerged fermentation has many advantages such as faster growth of mycelium (short cultivation cycle), higher efficient, lower cost, less pollution and higher content of bioactive metabolites than solid cultivation. In previous studies, the purpose of submerged fermentation for *C. militaris* is to obtain mycelial mass production and to extract bioactive metabolites from its mycelia and broth[12,13,14]. The submerged fermentation condition of *C. militaris* has a direct effect on the growth of mycelium, which includes the composition of carbon source and nitrogen source in fermentation medium, as well as suitable carbon and nitrogen ratio. On the other hand, fermentation conditions also have effects on the content of bioactive metabolites, such as cordycepin, adenosine and polysaccharide, etc. It has been reported that, in submerged cultivation of *C. militaris*, biochemical medium was used as fermentation medium[15,16]. Some indicated that natural materials could be used as components of fungal medium[17,18], which gives us revelation that natural materials may also be used to cultivate *C. militaris.* Nowadays most studies are focused on cordyceps polysaccharide in mycelium[19] and process optimization[20], few studies are about using submerged fermentation to produce cordycepin and cordycepic acid.

The purpose of this study was to optimize the submerged culture conditions to simultaneously produce mycelial biomass and bioactive components (i.e. cordycepin and adenosine) by *C. militaries* using a statistically-based experimental design. In the first step, one-factor-at-a-time method was used to investigate the effects of medium components (i.e. carbon, nitrogen, and mineral sources) and environmental factors (i.e. pH and temperature) on mycelial growth and bioactive components production. Subsequently, the combination of carbon and nitrogen sources of medium components was optimized using $L_9(3^4)$ orthogonal array (9 tests, 4 variables, 3 levels) method.

## MATERIALS AND METHODS

### Preparation of raw carbon and nitrogen sources

The rice, soybean and silkworm chrysalis used in this experiment, were purchased from a local farmers' market. The products were processed according to the following procedure. Weighed raw rice 10 g in a flask, and added an appropriate amount of water into the flask. Boiled the mixture for 15 min, and then filtered with eight layers of gauze. The filtered solution was diluted with water to the volume of 100mL for standby application. dealt with the raw soybean meal and silkworm chrysalis with the same method as the above description except for the heating the mixture in warm bath water of 80 °C for 2 h, and then filtered with eight layers of gauze.

### Microorganism and media

*C. militaris* strain was preserved by our laboratory. The stock culture was maintained on potato dextrose agar(PDA) slant and stored at 4°C. The solid medium, reformative potato dextrose agar (PDA) containing potato 200 g/L, dextrose 20 g/L, $MgSO_4 \cdot 7H_2O$ 1.5 g/L, $KH_2PO_4$ 2.5 g/L, $V_{B1}$ 10 mg/L, agar 20 g/L, was used for stock culture. The liquid medium was divided into seed medium and fermentation medium. The seed medium was consisted of potato 200 g/L, dextrose 20 g/L, $MgSO_4 \cdot 7H_2O$ 1.5 g/L, $V_{B1}$ 0.01 g/L and distilled water 1000 mL, with natural pH value, and then sterilized at 121°C for 30 min. The basal fermentation medium was consisted of glucose 35 g/L, peptone 5 g/L, yeast extract 5 g/L, $KH_2PO_4 \cdot H_2O$ 0.1 g/L, $MgSO_4 \cdot 7H_2O$ 0.5 g/L, $V_{B1}$ 0.05 g/L, and distilled water 1000 mL.

### Inoculum preparation

*C. militaris* was initially grown on PDA medium in a Petri dish, and then transferred to the

seed culture medium by punching out 10 mm of the agar plate culture with a sterilized self-designed cutter. The seed culture was grown in a 100 mL flask containing 20 mL basal medium at 25°C, 150 rpm for 5-6 d. The flask culture experiments were performed in a 250 mL flask containing 50 mL fermentation media after inoculating with 10% (v/v) of the seed culture.

**Fermentations**

Various fermentation media were inoculated with 10% (v/v) of the seed culture and then cultivated in a 250 mL flask containing 50 mL basal medium at 28°C on a rotary shaker incubator at 150 rpm for 72 h. Unless otherwise specified, fermentations were conducted under the following condition: temperature 28°C, shaking speed in the range of 120-150 rpm and with a natural initial pH. The seed culture was transferred to the fermentation medium and cultivated for 7 d. All experiments were performed at least in duplicate.

**Optimization of combination of several culture media on the growth of mycelia and production of bioactive components**

The combination of different media was studied by the growth of mycelia and production of bioactive component (adenosine and cordycepin), while compared with the effect of combinations of several culture media. Selection of combinations of the various culture media was tested by the $L_9$ ($3^4$) orthogonal design[21].

**Determination of mycelial biomass**

Samples collected at various intervals from flasks were centrifuged at 12000 rpm for 20 min, and the supernatant was discarded. The dry weight of mycelia were measured after repeated washing of the mycelial pellet with distilled water and drying at 40°C for overnight to a constant weight. The dry weight of mycelia was weighed and recorded respectively. All experiments contained three parallel tests.

**Determination of cordycepin and adenosine**

The sample of mecylium from each flask was dried at 40°C to a constant weight, and then grinded to powder with liquid nitrogen. Accurately weighed the powder 0.5 g, added 5 mL ethanol (50%, v/v) into it, ultrasonic treatment for 20 min, cycle for 3 times, and collected the extracting solution. The extracting solution was centrifuged at 12000 rpm for 20 min,and returned the supernatant for determination of the cordycepin and adenosine. Various samples were prepared to extract cordycepin and adenosine using previous reported method method[8]. Determination of the content of cordycepin and adenosine from the extraction was carried out by high-performance liquid chromatography (HPLC) method. All HPLC analysis work was carried out on a Waters 2695 Separation Module (Waters, Milford, MA, USA ), which consisted of a Waters 2996 Photo-diode Array Detector, an auto injector, and a reverse phase column (Waters Symmetry shield TM RP18/ 4.6 mm ×150 mm,5µm; Part No. 186000109). Standards of cordycepin and adenosine were purchased from Sigma Chemical Corporation (St. Louis, MO, USA). The determination condition of the samples was set as follows: the mobile phase adopted in the analysis consisted of water and methanol, which were in the ratio 88:12(V/V). The separation was conducted in isocratic elution with a flow rate of 1.00 mL/min. The detection wavelength of photo-diode array was set at 254 nm and the column temperature was 30°C. The injection volume was 10 µL [7,8]. All experiments contained three parallel tests. The results were expressed in means ± standard error (SE).

## RESULTS AND DISCUSSION

**Confirmation of culture time and initial pH**

In order to investigate the effects of culture time on the growth of mycelia, *C.militaris* was cultivated in 250 mL flask containing 50 mL basal fermentation medium at 28°C on a rotary shaker incubator at 150 rpm. Mycelial biomass (dry weight) was determined every 24 h after inoculation. The results showed that the mycelial biomass increased as the culture time went on (Fig. 1A). In view of *C.militaris* growing into stationary phases and accumulating metabolites, the suitable incubation time of *C.militaris* was confirmed on 5 d for further study.

Active mycelial growth was observed on the same medium at pH between 3.0 and 7.0 in the shake flasks. The results showed that the suitable pH for mycelial growth was between 5.0 and 7.0 (Fig.1B). Within this pH scale range, *C.militaris* grew well and formed into a regular mycelial pellet. As to the initial pH, investigators have different opinions, mainly common in neutral[22] and acidic[23,24,25] pH value. Based on the above results,

we chose pH 6.0 as initial pH condition for further optimization of medium composition for *C.militaris* growth and production of bioactive components.

**Effects of different carbon and nitrogen source on the growth of mycelia**

In order to investigate the suitable carbon source for the production of mycelia, various carbon sources, which replaced glucose of the

**Table 1.** Factors and levels of the orthogonal experiment (mg/L)

| Level | Factor | | | |
|---|---|---|---|---|
| | Soluble starch (A) | Fructose (B) | Soybean meal(C) | Silkworm chrysalis meal (D) |
| 1 | 22.50 | 12.50 | 5.00 | 2.50 |
| 2 | 17.50 | 17.50 | 3.75 | 3.75 |
| 3 | 12.50 | 22.50 | 2.50 | 5.00 |

**Table 2.** Analysis of the optimization results of mycelia biomass dry weight, adenosine, cordycepin in different culture media with the $L_9$ ($3^4$)orthogonal design (n=3)

| Item | | Factors | | | | Mycelia biomass dry weight(g) | Adenosine (μg/g) | Cordycepin (¼g/g) |
|---|---|---|---|---|---|---|---|---|
| | | A | B | C | D | | | |
| 1 | | 1 | 1 | 1 | 1 | 1.72±0.01 | 167.33±5.51 | 155.00±8.54 |
| 2 | | 1 | 2 | 2 | 2 | 2.36±0.67 | 188.00±18.19 | 161.00±25.06 |
| 3 | | 1 | 3 | 3 | 3 | 2.32±0.08 | 157.67±2.51 | 134.67±4.73 |
| 4 | | 2 | 1 | 2 | 3 | 1.25±0.23 | 170.67±14.29 | 178.00±28.51 |
| 5 | | 2 | 2 | 3 | 1 | 1.43±0.47 | 155.67±2.52 | 140.00±2.65 |
| 6 | | 2 | 3 | 1 | 2 | 2.15±0.60 | 167.33±3.21 | 143.00±4.00 |
| 7 | | 3 | 1 | 3 | 2 | 1.45±0.42 | 176.00±6.56 | 153.00±4.58 |
| 8 | | 3 | 2 | 1 | 3 | 1.85±0.29 | 172.33±7.57 | 243.33±116.90 |
| 9 | | 3 | 3 | 2 | 1 | 0.94±0.07 | 193.67±25.01 | 234.00±84.04 |
| Mycelia | $K_1$ | 6.40 | 4.42 | 5.72 | 4.09 | | | |
| biomass | $K_2$ | 4.83 | 5.64 | 4.55 | 5.96 | | | |
| | $K_3$ | 4.24 | 5.41 | 5.2 | 5.42 | | | |
| | $K_1/3$ | 2.13 | 1.47 | 1.91 | 1.36 | | | |
| | $K_2/3$ | 1.61 | 1.88 | 1.52 | 1.99 | | | |
| | $K_3/3$ | 1.41 | 1.8 | 1.73 | 1.81 | | | |
| | R | 0.72 | 0.41 | 0.39 | 0.63 | | | |
| Adenosine | $K_1$ | 513 | 513 | 506 | 515 | | | |
| | $K_2$ | 492 | 515 | 551 | 531 | | | |
| | $K_3$ | 541 | 518 | 489 | 500 | | | |
| | $K_1/3$ | 171 | 171 | 169 | 172 | | | |
| | $K_2/3$ | 164 | 172 | 184 | 177 | | | |
| | $K_3/3$ | 180 | 173 | 163 | 167 | | | |
| | R | 16 | 2 | 21 | 10 | | | |
| Cordycepin | $K_1$ | 451 | 486 | 541 | 529 | | | |
| | $K_2$ | 461 | 544 | 622 | 457 | | | |
| | $K_3$ | 630 | 512 | 573 | 556 | | | |
| | $K_1/3$ | 150 | 162 | 180 | 176 | | | |
| | $K_2/3$ | 154 | 181 | 207 | 152 | | | |
| | $K_3/3$ | 210 | 171 | 191 | 185 | | | |
| | R | 60 | 19 | 27 | 33 | | | |

K1/3, K2/3, K3/3 are for means of mycelia biomass, adenosine and cordycepin respectively, for the levels of each factor (n=3); R represents for range.

original basal medium, were provided at a concentration of 10 g/L for 5 days in the basal medium. On the whole, *C. militaris* grew well in the medium containing the 6 carbon sources respectively (Fig. 2A). In spite of this, there were still some differences, and the highest mycelial growth was obtained in soluble starch medium and fructose medium. Therefore, these two carbon sources were selected for optimization of medium composition for further study.

To investigate the suitable nitrogen sources on mycelial growth, fungal cells were cultivated in the medium containing various nitrogen sources, which replaced peptone and yeast extract of the original basal medium, were added to the basal medium at a concentration level of 10 g/L. Among 6 nitrogen sources examined, soybean meal, silkworm chrysalis meal, yeast powder and peptone were favorable to mycelial growth of *C. militaris* (Fig. 2B).

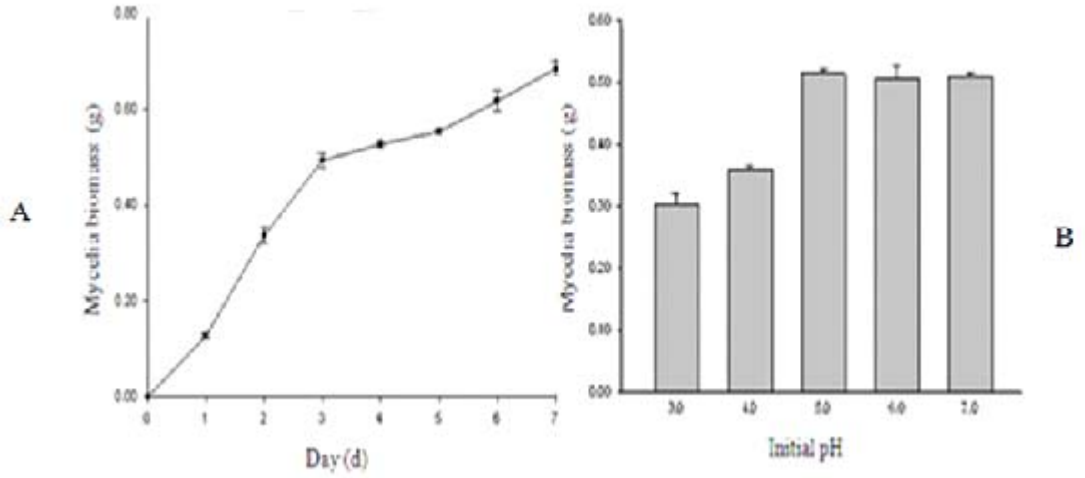


**Fig. 1.** Effect of culture time (A) and initial pH (B) on mycelial growth by *C. militaris* in shake flask cultures. Experimental data are mean ± S.D. of triple determinations

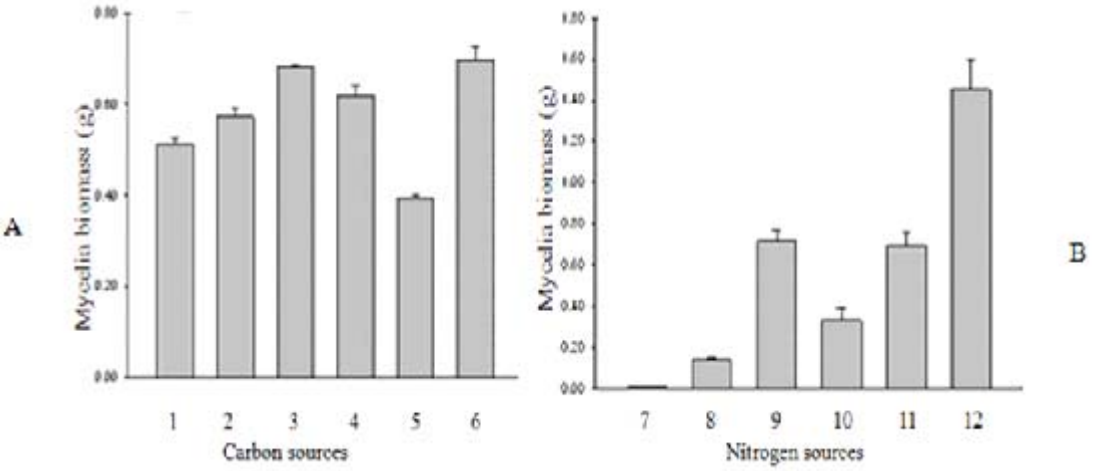


**Fig. 2.** Effect of carbon source (A) and nitrogen source (B) on mycelial biomass of *Cordyceps militaris*. 1-12 indicate sucrose, glucose, fructose, maltose, rice, soluble starch, urea, ammonis chloride, silkworm chrysalis meal, peptone, yeast and soybean meal, respectively.

**Effects of combinant of different carbon and nitrogen source on mycelial growth and production of bioactive components**

Based on the above-mentioned results, the nitrogen sources (soybean meal and silkworm chrysalis meal) were used for optimizing the medium combination of carbon and nitrogen source by a $L_9(3^4)$orthogonal array. In the present study, the requirements of kinds of carbon sources in the medium were not that strict to *C. militaris*[26]. Compared to carbon sources, the kinds of nitrogen resources was more important, and the organic nitrogen was easier to be utilized and promote the growth of mycelia than inorganic sources, perhaps due to the former contained many kinds of vitamins and other nutrients which were good to the fungal growth [27,28].

Good mycelial growth is the premise to obtain biologically active metabolite of *C. militaris*, and excellent combination of carbon and nitrogen sources provided material basis and energy for the growth and metabolites of *C. militaris.* On the basis of good growth of *C. militaris*, to get biologically active metabolites is the main purpose of cultured *C. militaris.* Therefore, the productions of adenosine and cordycepin as well as the mycelia biomass dry weight as the index were selected to screen the optimal combination of carbon and nitrogen source, and a conventional analytic method of $L_9$ ($3^4$) orthogonal arraywas employed. The levels of each factor for the $L_9$ ($3^4$) orthogonal array were shown in table 1, and the different combinations of carbon sources and nitrogen sources were listed in table 2. Variance analysis of $L_9$ ($3^4$) orthogonal experiment for the optimisation of mycelial biomass, adenosine and cordycepin was shown in table 3 respectively. The ranges of each factor A, B, C, D for mecylial biomass were 0.72, 0.41, 0.39, 0.63 respectively, the effect order was successive: soluble starch> silkworm chrysalis meal> fructose> soybean meal. The conclusion was that soluble starch was the most influential

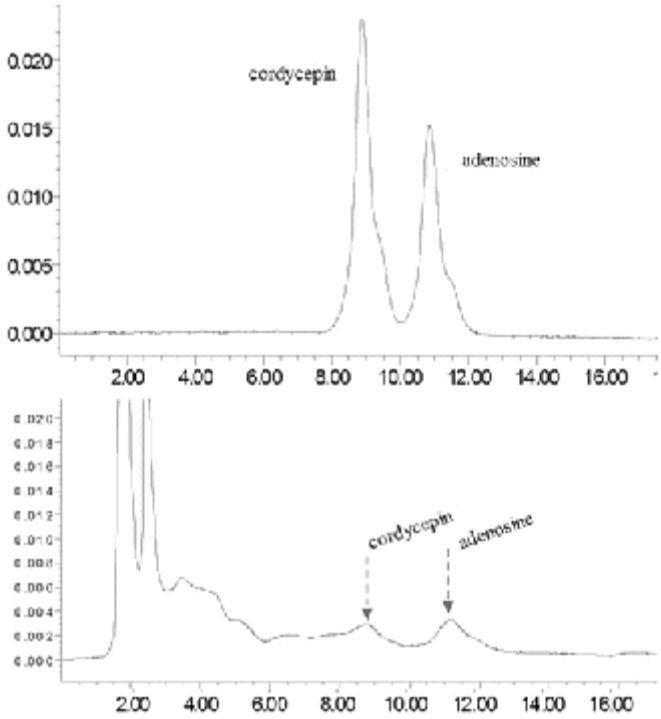


**Fig. 3.** HPLC chromatograms of ethanol extract of *C.militaris* mycelia (lower segment) and of chemiscal substance of both cordycepin and adenosine (upper segment). Retention time of adenosine and cordycepin of mixed standards under different percentage of water and methanol in mobile phase; water-methanol (88:12; V/V). Retention time of adenosine and cordycepin 8.9 and 11.2 min, respectively.Retention time of adenosine and cordycepin of sample water-methanol in mobile phase.

factor, the silkworm chrysalis meal took the second place, the fructose took the third place and the soybean meal was the subordination factor. Figure 3 showed that, along with levels of factors were increased, the average of the factor A was revealed gradually to decline trend, the factor B and D were revealed gradually to an upward trend early then to a decline trend, the factor C was revealed gradually to a decline trend early then to an upward trend. The result indicated that the optimal condition of accumulation of mycelial biomass dry weight was $A_1B_2C_1D_2$, soluble starch 22.50 g/L, fructose 17.50 g/L, soybean meal 5.00 g/L and silkworm chrysalis meal 3.75 g/L, with the accumulation of mycelial biomass reaching more than 2.36 g/L.

HPLC analysis was performed for determination of bioactive components,

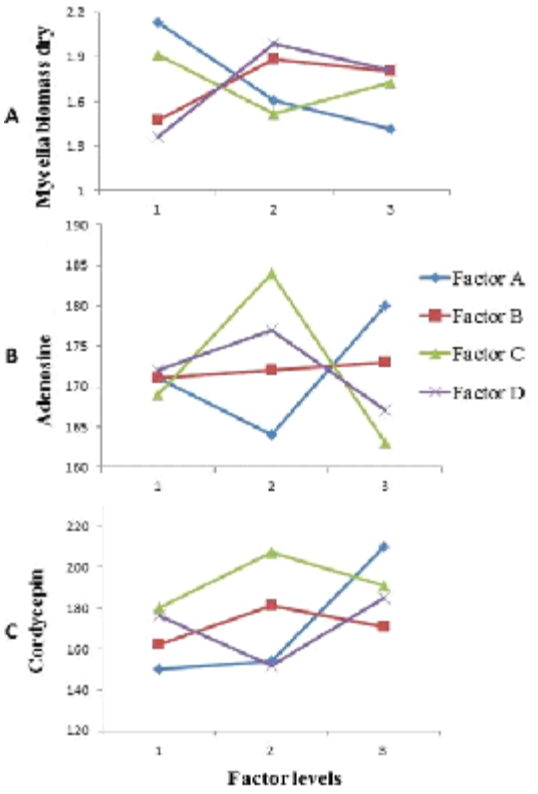


**Fig. 4.** The factors levels average index of $L_9$ ($3^4$) The Factor A, Factor B, Factor C, and Factor D indicate the average value of mycelia biomass dry weight (A), adenosine(B) andcordycepin(C) under the condition of soluble starch, fructose, soybean meal and silkworm chrysalis meal, respectively.

cordycepin and adenosine. Under an optimum chromatographic conditions, to the standards of cordycepin and adenosine, the retention time (Tr) was 8.960 and 11.23 min (shown in Fig. 4), limit of detection (based on a signal to noise ratio of 3:1) 0.25 and 0.30, processed channel (UV≈) 254.0, linear range 2.85-130 µg/mL and 2.50-120 µg/mL. Based on the chromatographic conditions, we established the standard curves cordycepin and adenosine standards (shown in supplementary Fig. 1). The regression equations of calibration curves and their coefficients were calculated as follows: for cordycepin, $Y=1.68661\times10^6$ X-92567.14024, R=0.9999 (n=5); for adenosine, $Y=2.5142\times10^6$ X-176688.96951, R=0.9999(n=5). Subsequently, the cordycepin and adenosine content of extracts from different fermentation samples was determinated by HPLC under same conditions (figure 4). HPLC analysis results showed that the contents of cordycepin and adenosine in the mycelia of *C.militaris* culturing on the different culture media composition was different. After being calculated using each regression equations of calibration curves, the data of cordycepin and adenosine content was used for further analysis.

As analysis in the same way as used for the optimal combination of carbon and nitrogen sources for mycelia biomass dry weight, the ranges of each factor A, B, C, D for adenosine were 16, 2, 21 and 10 respectively, the effect order was successive: soybean meal>soluble starch> silkworm chrysalis meal> fructose (shown in Fig. 3B). It showed that, along with levels of factors were increased, the average of the factor A was revealed gradually to a decline trend early then to an upward trend, the factor B was revealed gradually to an upward trend, the factor C and D were revealed gradually to an upward trend early then to a decline trend. The result indicated that the optimal condition of production of cordycepin was $A_3B_3C_2D_2$, soluble starch 12.50 g/L, fructose 22.50 g/L,soybean meal 3.75 g/L and silkworm chrysalis meal 3.75 g/L, under which condition the accumulation of adenosine would reach more than 193.67 µg/g. It was the same method used in the analysis of production of cordycepin. The optimal condition of production of adenosine was $A_3B_2C_2D_3$, soluble starch 12.50 g/L, fructose 17.50 g/L, soybean meal 3.75 g/L and silkworm chrysalis meal 5.00 g/L(shown in Fig. 3C), under which

condition the accumulation of cordycepin would reach more than 243.33 µg/g. The optimal combination of carbon and nitrogen sources for adenosine and cordycepin were nearly same, except for factor D and factor B, since factor B was the forth subordinate factor for the production of both adenosine and cordycepin, and factor D was the third subordinate factor for the production of adenosine and the second subordinate factor for the production of cordycepin. It implied that the effect of the carbon and nitrogen sources in the medium on the production of adenosine and codycepin was almost same, and they were both the metabolic products of *C. militaris* after it grew into stable phase.

The character of a given physiological and biochemical process occurring in microorganism can be elucidated only by cultivating them on selected media. Selection of suitable medium composition for cultivation of microorganism is very important to reveal their potential ability to produce various bioactive compounds. In an overview of previous literatures, there was a large collection of papers on optimization of culture conditions for mycelial growth and production of polysaccharide and cordycepin[30-38]. The reason why *C. militaris* draws so much attention is that it is a potential substitute of *O.sinensis*, which plays an important role in disease prevention and treatment in folk medicine[19], and cordycepin is usually produced synthetically at present. The experimental studies carried out to evaluate cultural requirement for *C. militaris* have provided good information on the effects of test parameters upon mycelial biomass on liquid state culture. Nutritional modifications such as carbon sources, nitrogen sources, and combination of carbon and nitrogen sources affect the mycelial biomass and production of cordycepin and adenosine.

## CONCLUSION

It has been shown in this study how a culture medium for production biomass, adenosine and cordycepin by using *C.militaris* was optimized by using a statistical design strategy combining analysis of variance. To different bioactive components, the optimized medium is various, e.g., mycelial biomass was obtained in the optimized medium which consisted of 22.50 g/L soluble starch, 17.50 g/L fructose, 5.00 g/L soybean meal and 3.75 g/L silkworm chrysalis meal, while adenosine production was obtained in the optimized medium which was consisted of 12.50 g/L soluble starch, 22.50 g/L fructose, 3.75 g/L soybean meal and 3.75 g/L silkworm chrysalis meal, and cordycepin production was obtained in the optimized medium which consist of 12.50 g/L soluble starch, 17.50 g/L fructose, 3.75 g/L soybean meal and 5.00 g/L silkworm chrysalis meal. This showed, once more, that these methods are very useful for optimizing fermentation, and in the specific case of *C. militaris*, optimizing processes for producing it and its metabolites in submerged culture as a sound alternative to traditional culture.

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