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Contributed Paper

Optimization of Solid-state Fermentation for Fruiting Body Growth and Cordycepin Production by *Cordyceps militaris*

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ABSTRACT

Cordyceps sinensis has traditionally been used in Traditional Chinese Medicine, however *C. militaris* has gained importance and is also used as a functional food. *Cordyceps militaris* contains similar biochemical components as *C. sinensis* as well as the anti-cancer component cordycepin. Because *C. militaris* can be grown in culture and has significant medicinal affects, market demand for artificial *C. militaris* has increased. This study was aimed to optimize the conditions of solid-state fermentation for fruiting body growth and cordycepin production from *C. militaris* by one-factor-at-a-time and orthogonal layout methods. The optimal culture substrate was brown rice. The optimal fruiting body growth and cordycepin production were observed at relatively low pH value. The optimum composition were 40 g/L glucose, 5 g/L peptone, 1.5 g/L $MgSO_4 \cdot 7H_2O$, 1.5 g/L K_2HPO_4 and 1.0 mg/L NAA for optimal fruiting body growth and 10 g/L glucose, 10 g/L peptone, 1.0 g/L $MgSO_4 \cdot 7H_2O$, 1.0 g/L K_2HPO_4 and 1.0 mg/L NAA for cordycepin production. These optimization strategies in solid medium culture lead to a 67.96 % (1.73±0.08 g/bottle) increase in fruiting body yield and a 63.17% (9.17±0.09 mg/g) increase of cordycepin yield in fruiting body, which may be applied in industrial production of cordycepin via solid-state fermentation.

Keywords: *Cordyceps militaris*; solid-state fermentation, fruiting-body, cordycepin, optimization

1. INTRODUCTION

The genus *Cordyceps* Fr. (Clavicipitaceae, Hypocreales, Ascomycota) comprising over 400 species and varieties is now divided into the families *Cordycipitaceae*, *Ophiocordycipitaceae* and *Clavicipitaceae* [1]. Most members of this group are pathogenic on different insects, spiders, while a few grow on hypogeal fungi of *Elaphomyces* spp. Many taxa are used as an invigorant in Traditional Chinese Medicine (TCM). *Cordyceps militaris*(L.) Link is one of the most important species of the *Cordyceps* group, which generally parasitizes larva or pupa of lepidopteran insects and forms fruiting bodies on their insect hosts.

Currently *C. militaris* is used as functional food and medicine in Southeast Asia [23]. This species may become a medicinal fungus with the largest production and popular usage in the future. *Cordyceps militaris* is now used as a substitute of *C. sinensis* in TCM as well as health foods, as the latter is hugely expensive [40]. In comparison with *C. sinensis*, *C. militaris* contains similar bioactive components but a greater quantity of cordycepin [2]. Because of its significant pharmacological activity, the market demand of C. *militaris* has increased [41].

Cordyceps militaris possesses extensive bioactive compounds including polysaccharides, cordycepin and ergosterol with significant pharmacological effects [3]. In recent studies, haemagglutinin [4] and a cytotoxic antifungal protease were purified from the dry fruiting body of *C. militaris* [5]. Cordycepin, a nucleoside derivative, isolated from the culture liquid of C. *militaris* has drawn considerable interest [6], and has been found to have antitumor [7], antivirus [8], antileukemi [9] and hypolipidemic [10] properties, and in treating and prevention of obesity [11]. Cordycepin is also a Phase I/II clinical stage drug candidate for the treatment of refractory Acute Lymphoblastic Leukemia (ALL) patients who express the enzyme terminal deoxynucleotidyl transferase (TdT) (July, 2008. OncoVista-NCT00709215). Recently, research has shown that many of the reported bioactive effects of cordycepin are likely to be due to its effects on mTOR (mammalian target of rapamycin) and AMPK (AMP-activated kinase) signaling [12].

Methods for the synthesis of cordycepin are via chemical and biological pathways. Since cordycepin obtained by chemical pathways is difficult to purify and the cost is much higher than the biological pathways, the major research concern is the biological pathways [13]. The difficulty in producing secondary metabolites comes from the lack of knowledge of interactions between environment and microorganism [14]. There have been many studies on culture requirements for secondary metabolite production of filamentous fungi [15,16]. Similarly, *in vitro* mycelium growth and fruiting body formation of *C. militaris* have attracted the interests from mycologists, entomologists and biotechnologists. There have been studies on the culture condition [17-19] and medium composition [20-24] for increasing the yield of cordycepin in liquid culture. There are only a few reports on the solid-state fermentation of *C. militaris*. Fruiting body formation [25], cordycepin production in medium [26] and the optimum solid substrate [27] of cordycepin production in fruiting bodies in *C. militaris* by solid-state fermentation has been reported. However cordycepin content in these fruiting bodies of *C. militaris* was relatively low. There has been no investigation optimizating media to simultaneously improve fruit body formation and cordycepin production using solid-state fermentation.

The objective of this study was to optimize solid-state fermentation of *C. militaris* in order to increase yields of fruiting bodies and cordycepin via a statistically based experimental design. Medium optimization (substrate and nutritional solution) by a one-factor-at-a-time method which involved changing one independent variable at a time (i.e. nutrient, and pH). Hence as a more practical method, the orthogonal matrix method was employed to study the relationships between the medium components and their effects on fruiting body formation and cordycepin production.

2. MATERIALS AND METHODS

2.1 Microorganism and Inoculum Preparation

The isolate of *C. militaris* CGMCC2459 used in the present study was collected from Mt. Qingcheng in Sichuan Province. The stock

culture was maintained on potato dextrose agar (PDA) slant. The culture was inoculated onto slants and incubated at 26°C for 7 d. Six ml of sterilized distilled water was added to the slant and spores washed off and then filtered through sterilized absorbent cotton in infundibulum. The liquid filtrate containing spores of *C. militaris* was added into seed culture medium with a suitable concentration (optimal concentration is 3×10^8 spores/ml, the number of spores was counted using Thoma's hematocytometer). The seed culture was grown in a 250 ml flask containing 50 ml of basal medium (20 g/L sucrose, 20 g/L peptone, 0.5 g/L $MgSO_4\cdot7H_2O$ and 1g/L K_2HPO_4) at 23°C on a rotary shaker incubator at 150 rev/min for 4 d.

2.2 Solid-state Fermentation for Fruiting

Fruiting medium of *C. militaris* was prepared by mixing 20 g of rice (or other substrates) and 32 ml of nutritional solution (20 g/L sucrose, 10 g/L peptone, 0.1 g/L $MgSO_4\cdot7H_2O$ and 0.1 g/L KH_2PO_4 with 1,000 mL distilled water) in a 300 mL cylindrical glass bottle (8 cm in diameter and 12 cm in height) and then sealed with plastic and were autoclaved for 30 min at 121°C. The medium was cooled to room temperature and inoculated with 5 mL seed culture and incubated at 20°C for 12 d and was given dark treatment for promoting vegetative growth. Primordia of fruiting bodies began to form at 12-15 d after lowering the incubation temperature to 16°C at night (darkness) with culture temperature maintained at 23°C during the day (the white light maintained at 500 lx) and relative humidity (RH) at 90%-95%. While the temperature was maintained at 23°C and RH at 80%-90%, sufficient air exchanges were used to maintain CO_2 levels. Illumination with 300 lx intensity did not exceed 12 hours per day. The culture developed into 5-9 cm long fruiting bodies within 50-60 d following inoculation. All experiments were performed at least in duplicate.

The basal solid substrates tested for fruiting body and cordycepin production in solid-state fermentation included brown rice, millet, sorghum, corn, wheat or glutinous rice. Different carbon and nitrogen sources, mineral salts, and growth factors on the effect of solid-state fermentation on fruiting body growth and cordycepin production were also compared using one-factor-at-a-time and orthogonal layout methods.

2.3 Growth Characteristics in Batch Culture

To investigate the fermentation kinetics on fruiting body growth and cordycepin production, *C. militaris* was cultivated in 300-ml cylindrical glass bottles under the following conditions: Fruiting medium of *C. militaris* was prepared by mixing 20 g of rice and 32 mL of nutritional solution (10 g/L glucose, 10 g/L peptone, 1 g/L $MgSO_4\cdot7H_2O$, 1 g/L K_2HPO_4 and 1 mg/L NAA with 1,000 mL distilled water, pH 6.0) in a cylindrical glass bottle and autoclaving for 30 min at 121°C. Each glass bottle containing fruiting medium was inoculated with 5 mL of liquid inoculum of *C. militaris* for *in vitro* fruiting. After inoculation, the bottles were incubated at 20°C under dark for 12 days. Primordia of fruiting-bodies began to form after lowering the incubation temperature to 16°C at night (darkness) with the temperature maintained at 23°C during the day (the light maintained at 500 lx) and relative humidity (RH) at 90%-95% for 8 d. At last, under 14:10 L:D (300 lx light) at 23°C and high humidity conditions (80-90%) for 40 days, the culture develops 5-9 cm long fruiting-bodies.

2.4 Analytical Methods

The fruiting body was dried to a constant weight at 55°C overnight. Cordycepin and

adenosine in fruiting bodies were analyzed by high-performance liquid chromatography (1,100 series, Agilent Technology, U.S.). Standard cordycepin and adenosine (from Sigma) were dissolved in distilled water for calibration. The mobile phase was 10 mM KH_2PO_4, which was dissolved in methanol/distilled water (6:94). Elution was performed at a flow rate of 1 ml/min with column temperature at 45°C and the UV wavelength of 259 nm.

2.5 Statistical Analysis

All data obtained before by variance analysis. Differences of $F > 0.10$ or $F < 0.05$ or $F > 0.01$ were considered different significant levels.

3. RESULTS AND DISCUSSION

3.1 One-factor-at-a-time Method

3.1.1 Effect of Different Solid Substrates

In this study, the basal solid substrates for fruiting body and cordycepin production in solid-state fermentation including brown rice, millet, sorghum, corn, wheat or glutinous rice was tested. Mycelia entirely colonized 300 ml bottles containing 20g of basal substrate medium within 12 d following inoculation. The brown rice was found to be the best basal substrate for fruiting body and cordycepin production. The highest yield of fruiting body was 1.03±0.08 g/bottle. Amylopectin-rich grain including millet, sorghum and glutinous rice were not such good media for fruiting body production (Table 1). *C. militaris* may find it difficult to utilize Amylopectin, furthermore these grains stuck to each other after sterilization, so that gas permeability was likely reduced which may not beneficial for fungal growth.

Stromata (fruiting-body) formation of *C. militaris* on *Mamestra brassicae* pupae via percutaneous infection using ascospores has been previously reported [28]. *C. militaris* fruiting body production was tested via injecting a suspension of its hypha into pupae of three lepidopteran species; *Mamestra brassicae*, *Spodoptera litura* and *Bombyx mori* and a coleopteran species *Tenebrio molitor*. All of the pupae required a shorter period for stromata formation [29]. However this method was not cost effective for industrial production. Therefore, the use of low-cost grain to investigate fruiting body and cordycepin production is important. The results obtained in our study differ from those using optimum solid substrates to produce *C. militaris* fruiting bodies and cordycepin which found wheat [30] and soybean [27] were optimal.

Table 1. Effect of different basal substrates on fruiting body and cordycepin production.

Basal substrate	Fruiting body dry weight (g/bottle)	Adenosine content in fruiting bodies (mg/g)	Cordycepin content in fruiting bodies (mg/g)
Brown rice	1.03±0.08	0.61±0.03	5.62±0.03
Millet	0.13±0.01	1.07±0.05	3.34±0.12
Sorghum	0.15±0.06	1.45±0.22	3.81±0.06
Corn	0.24±0.06	1.12±0.11	2.59±0.14
Wheat	0.56±0.04	0.83±0.02	5.20±0.11
Glutinous rice	0.16±0.01	1.14±0.14	2.42±0.08

*Initial pH of fermentation was 5.50. Results are means of three replicates, standard deviations are also indicated.

3.1.2 Effect of Different Carbon and Nitrogen Sources

To investigate the effect of carbon sources on fruiting body and cordycepin production (Table 2), glucose, sucrose, amidulin, lactose, maltose and mannose were tested. The amidulin medium produced the highest fruiting body yield followed by glucose medium. On the other hand, glucose was better for cordycepin accumulation than amidulin. When consideration the cost of these additives, glucose is recommended for large scale industrial fruiting body and cordycepin production.

Amongst the six nitrogen sources added to the basal medium at a concentration level of 10 g/L (Table 2), peptone was the best for fruiting body production. However, the maximal cordycepin production was achieved with soybean oil meal. When considering fruiting body and cordycepin production, peptone is a desirable nitrogen source.

Similar observation was reported by other researchers for other *Cordyceps* spp. [31-33]. Carbohydrates are important carbon and energy sources for cultured cells. The results concerning the carbon source in this study are in agreement with other reports for *C. unilateralis* and *C. takaomontana* [32,33]. This study showed that peptone as the nitrogen source, increased fruiting body and cordycepin production this differs from previous reports [32,33]. Furthermore the optimum nitrogen source to produce cordycepin by *C. militaris* in submerged culture was YE [19]. This analogy may result from different fermentation methods.

Table 2. Effect of carbon and nitrogen sources on the fruiting body and cordycepin production by *C. militaris**.

	Fruiting body dry weight (g/bottle)	Adenosine content in fruiting body (mg/g)	Cordycepin content in fruiting body (mg/g)
Carbon source			
Glucose	1.36±0.05	1.12±0.06	6.50±0.06
Sucrose	1.29±0.04	0.90±0.03	4.03±0.03
Amidulin	1.39±0.02	1.03±0.04	6.21±0.13
Lactose	0.65±0.07	0.74±0.03	5.09±0.07
Maltose	0.28±0.03	0.19±0.02	3.77±0.04
Mannose	0.64±0.04	0.92±0.06	5.84±0.06
Nitrogen source			
Wheat bran	1.32±0.01	1.08±0.03	7.06±0.09
Soybean oil meal	0.16±0.03	0.66±0.05	10.90±0.04
Beef extract	1.06±0.03	0.10±0.09	2.82±0.05
Peptone	1.75±0.07	1.12±0.13	6.13±0.04
Yeast extract	1.55±0.06	1.15±0.03	1.78±0.06
Silkworm pupa	1.43±0.04	0.91±0.11	5.51±0.08
NH_4NO_3	1.18±0.03	0.90±0.08	3.15±0.04

*Initial pH of fermentation was 5.50. Results are means of three replicates, standard deviations are also indicated.

3.1.3 Effect of Different Mineral Salts

Mineral salts have been reported to be important for growth and development of different fungi [33-36]. Therefore, salt components in basal medium (nutritional solution) were included in this study. The effect of various mineral salts at the concentration level of 0.1 g/L on fruiting body and cordycepin production was examined. Amongst the mineral salts tested, K_2HPO_4 and $MgSO_4 \cdot 7H_2O$ yielded good fruiting body and cordycepin production (Table 3).

Table 3. Effect of different mineral salts on fruiting body and cordycepin production*.

Mineral sources	Fruiting body dry weight (g/bottle)	Adenosine content in fruiting body (mg/g)	Cordycepin content in fruiting body (mg/g)
Control†	0.49±0.03	1.12±0.05	3.91±0.08
K_2HPO_4	1.42±0.01	1.04±0.02	5.72±0.04
KH_2PO_4	1.30±0.04	0.81±0.01	5.31±0.03
$Ca(NO_3)_2$	0.53±0.04	0.75±0.01	5.09±0.11
$CaCl_2$	0.32±0.03	1.23±0.04	5.55±0.07
KCl	0.23±0.01	0.91±0.02	3.80±0.01
$MgSO_4 \cdot 7H_2O$	1.38±0.03	1.17±0.03	5.60±0.11
$FeSO_4$	0.05±0.01	0.66±0.02	0.95±0.03

*Initial pH of fermentation was 5.50. Results are means of three replicates, standard deviations are also indicated.

†Control means no supplementation of mineral salts.

3.1.4 Effect of Different Growth Factors

In order to find the best growth factor for fruiting body growth and cordycepin production, *C. militaris* was cultured in a basal medium (nutritional solution) with different vitamins and plant growth hormones in solid-state fermentation. All the plant growth hormones and vitamins tested increased fruiting-body production (Table 4). However, only α-naphthylacetic acid (NAA) achieved the highest yield of fruiting body and cordycepin production. This result is in agreement with our previous study on cordycepin production in submerged culture of *C. militaris*[37].

3.1.5 Effect of Initial pH

The pH of media is a very important but is often a neglected environmental factor. In this study, the maximum fruiting body growth of 1.81±0.08 g and the maximum cordycepin production of 7.40 ±0.01 mg/g were achieved at pH 5.5-6.0 (Table 5). This is similar to the result for cordycepin production by *C. militaris* in submerged culture[19,37]. Previous studies have also shown that the growth of entomopathogens *Beauveriabassiana*, *Metarhiziumanisopliae* and *Paecilomycesfarinosus* was optimal from pH 5 to 8[38].

3.2 Orthogonal Matrix Method

To investigate the relationships between variables of nutritional solution components and optimize their concentrations for fruiting body growth and cordycepin production, the orthogonal matrix L_{16} (4^5)

Table 4. Effect of different growth factors on fruiting body and cordycepin production*.

Growth factor	Fruiting body dry weight (g/bottle)	Adenosine content in fruiting body (mg/g)	Cordycepin content in fruiting body (mg/g)
Control†	1.05±0.03	1.12±0.02	4.40±0.05
Vitamin B_1 (VB_1)	1.15±0.03	1.16±0.01	2.92±0.04
Vitamin B_9 (VB_9)	1.50±0.07	1.11±0.02	3.55±0.03
α-naphthylacetic acid (NAA)	1.32±0.04	0.78±0.01	6.21±0.08
2,4-Dichlorophenoxyacetic acid (2,4-D)	1.15±0.04	0.93±0.03	4.12±0.21
Indole-3-butytric acid (IBA)	1.36±0.01	0.82±0.02	3.19±0.11

*Initial pH of fermentation was 5.50. Results are means of three replicates, standard deviations are also indicated.

†Control means no supplementation of growth factor.

Table 5. Effect of initial pH on fruiting body and cordycepin production*.

pH value	Fruiting body dry weight (g/bottle)	Adenosine content in fruiting body (mg/g)	Cordycepin content in fruiting body (mg/g)
5.0	1.47±0.04	0.71±0.02	3.80±0.06
5.5	1.81±0.08	0.83±0.07	5.29±0.13
6.0	1.61±0.13	1.07±0.08	7.40±0.01
6.5	1.59±0.07	1.21±0.02	7.21±0.04
7.0	1.52±0.01	1.02±0.04	6.53±0.03
7.5	1.41±0.01	1.04±0.01	5.51±0.03
8.0	1.45±0.07	1.30±0.06	4.39±0.01

*Results are means of three replicates, standard deviations are also indicated.

method was used. According to the above results achieved using one-factor-at-a-time, we selected and separated four levels as shown in Table 6. The experimental conditions and results for each project are listed in Table 7. The fermentation conditions of initial pH, inoculum volume and growth period were fixed to be 6.0, 5 mL/bottle and 55 days.

The highest mean yield of fruiting bodies was 1.68±0.11 g/bottle obtained from the ninth run group (Table 7 and Figure 1). The levels of corresponding factors involved A3, B1, C3, D4 and E2, namely glucose (30 g/L), peptone (5 g/L), $MgSO_4 \cdot 7H_2O$ (1.5 g/L), K_2HPO_4 (2.0 g/L) and NAA (1.0 mg/L). Whereas, maximum cordycepin production in fruiting bodies of 7.84±0.03 mg/g was found in the second run group with the levels of corresponding factors including glucose (10 g/L), peptone (10 g/L), $MgSO_4 \cdot 7H_2O$ (1.0 g/L), K_2HPO_4 (1.0 g/L) and NAA (1.0 mg/L) (A1B2C2D2E2).

Table 6. Experimental factors and their levels for orthogonal layout L_{16} (4^5).

Level	Glucose (A) g/L	Peptone (B) g/L	$MgSO_4 \cdot 7H_2O$ (C) g/L	K_2HPO_4 (D) g/L	NAA (E)mg/L
1	10	5	0.5	0.5	0.5
2	20	10	1.0	1.0	1.0
3	30	15	1.5	1.5	1.5
4	40	20	2.0	2.0	2.0

Table 7. Results of L_{16} (4^5) orthogonal layout for fruiting body growth and cordycepin production*.

Run	A	B	C	D	E	Fruiting body dry weight (g/bottle)	Adenosine content in fruiting body (mg/g)	Cordycepin content in fruiting body (mg/g)
1‡	1†	1	1	1	1	1.32 ± 0.06	1.67 ± 0.02	4.82 ± 0.12
2	1	2	2	2	2	1.32 ± 0.03	1.64 ± 0.05	7.84 ± 0.03
3	1	3	3	3	3	1.11 ± 0.07	1.43 ± 0.13	6.01 ± 0.12
4	1	4	4	4	4	0.50 ± 0.02	1.20 ± 0.07	4.86 ± 0.07
5	2	1	2	3	4	1.61 ± 0.04	1.47 ± 0.02	3.73 ± 0.01
6	2	2	1	4	3	0.54 ± 0.03	1.43 ± 0.05	6.30 ± 0.06
7	2	3	4	1	2	0.78 ± 0.01	1.96 ± 0.01	6.09 ± 0.09
8	2	4	3	2	1	0.53 ± 0.05	1.71 ± 0.04	5.02 ± 0.04
9	3	1	3	4	2	1.68 ± 0.11	1.60 ± 0.02	5.70 ± 0.03
10	3	2	4	3	1	0.80 ± 0.06	1.50 ± 0.03	4.83 ± 0.05
11	3	3	1	2	4	0.58 ± 0.02	1.24 ± 0.11	5.71 ± 0.03
12	3	4	2	1	3	0.35 ± 0.01	1.08 ± 0.02	5.40 ± 0.11
13	4	1	4	2	3	1.52 ± 0.09	1.22 ± 0.06	4.23 ± 0.08
14	4	2	3	1	4	1.28 ± 0.03	1.43 ± 0.03	5.43 ± 0.04
15	4	3	2	4	1	0.89 ± 0.02	1.48 ± 0.02	5.91 ± 0.07
16	4	4	1	3	2	0.58 ± 0.04	0.99 ± 0.03	5.64 ± 0.13

*Results are means of three replicates, standard deviations are also indicated.

†The arrangements of column A–E were decided by orthogonal design for L_{16} (4^5).

‡Every row of run number represents one experimental replicate, and every run was replicated thrice.

The effect of media on fruiting body growth and cordycepin production was calculated according to the orthogonal method (Table 8). In accordance with the magnitude order of R (Max Dif), the order of effect of all factors on fruiting body growth could be determined. The order of effects of factors on fruiting body growth was peptone > K_2HPO_4 > glucose > NAA > $MgSO_4 \cdot 7H_2O$. By applying the same method, the order of effects of factors on cordycepin production in fruiting bodies were peptone > NAA > K_2HPO_4 > $MgSO_4 \cdot 7H_2O$ > glucose.

To obtain the optimum composition of each factor, the maximum K value of each column based on statistical calculation using the data in Table 7 was calculated

Table 8. Analysis of the effect of nutritional solution composition on fruiting body growth and cordycepin production of *C. militaris* via SSF with orthogonal test.

	Fruiting body dry weight (g/bottle)					Cordycepin content in fruiting body (mg/g)				
	A	B	C	D	E	A	B	C	D	E
K_1	4.25*	6.13	3.02	3.73	3.54	23.53	18.48	22.47	21.74	20.58
K_2	3.46	3.94	4.19	3.95	4.36	21.14	24.40	22.88	22.80	25.27
K_3	3.41	3.36	4.60	4.10	3.52	21.64	23.72	22.16	20.21	21.94
K_4	4.27	1.96	3.60	3.61	3.97	21.21	20.92	20.01	22.77	19.73
k_1	1.06†	1.53	0.76	0.93	0.89	5.88	4.62	5.62	5.44	5.15
k_2	0.87	0.99	1.04	0.99	1.09	5.29	6.10	5.72	5.70	6.32
k_3	0.85	0.84	1.15	1.03	0.88	5.41	5.93	5.54	5.05	5.49
k_4	1.07	0.49	0.90	0.90	0.99	5.30	5.23	5.00	5.69	4.93
R	0.21‡	1.04	0.40	0.12	0.21	0.60	1.48	0.72	0.65	1.38
Optimal level	4	1	3	3	2	1	2	2	2	2

* $K_i^A = \Sigma$ Fruiting body yield at A*i*. Values are mean of triple determinations.

† $k_i^A = K_i^A/3$. Values are mean of triple determinations.

‡ $R_i^A = \max\{K_i^A\} - \min\{k_i^A\}$. Values are mean of triple determinations.

(Table 9). The results were as follows: (1) to obtain a high fruiting body biomass, the optimum nutritional solution composition is glucose (40 g/L), peptone (5 g/L), $MgSO_4 \cdot 7H_2O$ (1.5 g/L), K_2HPO_4 (1.5 g/L) and NAA (1.0 mg/L) (A4B1C3D3E2). (2) To obtain a high cordycepin production in the fruiting-bodies, the optimum nutritional solution composition is glucose (10 g/L), peptone (10 g/L), $MgSO_4 \cdot 7H_2O$ (1.0 g/L), K_2HPO_4 (1.0 g/L) and NAA (1.0 mg/L) (A1B2C2D2E2).

The orthogonal layout method is one of the most important statistical methods using Taguchi parameter design methodology [39]. It is feasible to investigate the influence of controlled factors in a multivariable system and give effective responses in the course of system optimization. Therefore, this method has been widely applied in industry. In the present study, the one-factor-at-a-time method was employed to observe effects of variables of medium constituents and culture conditions on fruiting body growth and cordycepin production. Each of the nutritional solution components was subsequently optimized using the orthogonal design. The effect of peptone on the cordycepin production was more important than that of other nutrients, and the effect of peptone on fruiting body growth was highly significant.

Table 9. The variance analysis of the results of L_{16} (4^5) orthogonal test for fruiting body growth and cordycepin production.

Variance source	Fruiting body dry weight (g/bottle)				Cordycepin content in fruiting body (mg/g)			
	Sum of square deviation (SS)	Degree of freedom (v)	Mean square (MS)	*F* ratio and significance level	Sum of square deviation (SS)	Degree of freedom (v)	Mean square (MS)	*F* ratio and significance level
A	0.17	3	0.057	4.75	0.94	3	0.31	1.00
B	2.26	3	0.75	62.64**	5.55	3	1.85	5.88
C	0.35	3	0.60	9.83*	1.23	3	0.41	1.30
D	0.04	3	0.012	1.00	1.11	3	0.37	1.18
E	0.12	3	0.04	3.33	4.45	3	1.48	4.71
e†	0.04	3	0.012	-	0.13	3	0.31	-

*F raito > $F_{0.05}$, $F_{0.05}(3,3) = 9.28$.

**Fratio > $F_{0.01}$, $F_{0.01}(3,3) = 29.50$.

†*e* means error.

Figure 1. L_{16} (4^5) orthogonal layout for fruiting body growth and cordycepin production for 55 days.

3.3 Growth Characteristics in Batch Culture

As shown in Figure 2, during the entire fermentation period, the media dry weight declined at all times from 18.82 to 13.67. Changes in fruiting body and cordycepin content in fruiting bodies showed similar kinetic curves. Fruiting body and cordycepin content in fruiting bodies increased sharply after the lag growth phase and before the exponential growth metaphase, increasing and fluctuating slightly until fermentation was terminated. The results were similar to kinetics profiles of cordycepin fermentation in *C. militaris* by submerged culture [18, 19] and surface culture[18,21], but differed from cordycepin production in media by solid-state fermentation for *C. militaris* [26].

Adenosine content in fruiting bodies was small. It increased slightly from 0.23±0.01 mg/g at 16 d, and then rose slowly in succession, reached around 2.67±0.04 mg/g during the later stages of fermentation, and fluctuated slightly until fermentation was terminated. The maximum fruiting body number was 1.73±0.08 g/bottle after 60 days of fermentation, but the maximum cordycepin content in fruiting bodies was 9.17±0.09 mg/g (this was 63.17% higher than before the optimization of culture requirements) after 72 days of fermentation. In considering the energy and fermentation time costs, a 60-day period (cordycepin content in fruiting bodies was 8.71±0.15 mg/g) is suitable for simultaneous higher production of fruiting bodies and cordycepin content in fruiting bodiesof *C. militaris*by solid-state fermentation.Fruiting bodiesappeared somewhat withered after 60 days indicating that it was a suitable termination time (Figure 1).

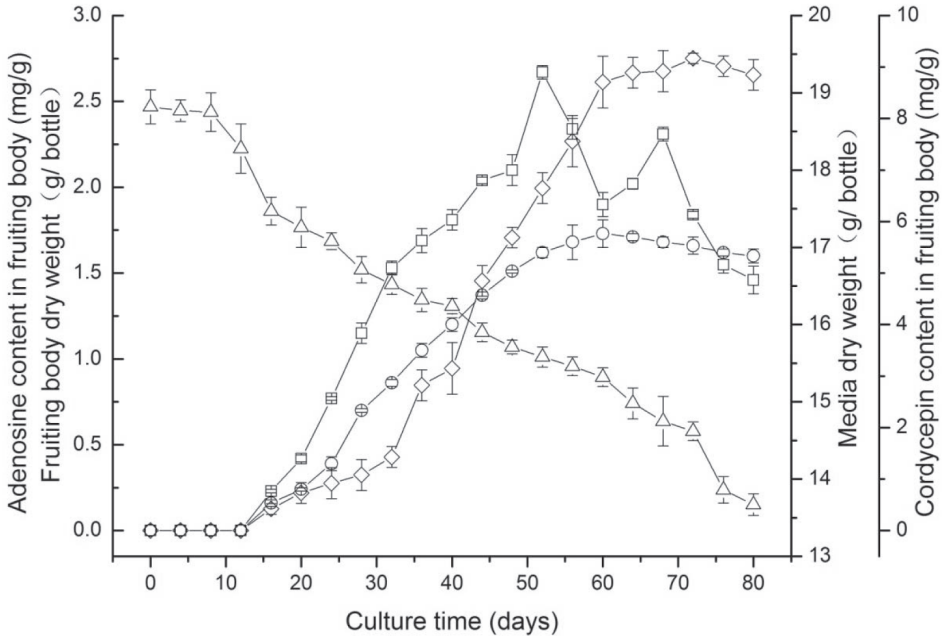

Figure 2. The solid-state fermentation period of *C. militaris* in batch cultures under optimal culture conditions: (○) fruiting body dry weight, (△) media dry weight, (□) adenosine content in fruiting body, (◇) cordycepin content in fruiting body.

4. CONCLUSIONS

This optimization strategy in solid-state fermentation resulted in increase of fruiting body yield to 67.96% (1.73±0.08 g/bottle) and cordycepin content in fruiting body to 63.17%(9.17±0.09 mg/g). The results obtained in this work could have a significant impact on industrial scale production of fruiting bodiesand cordycepin by solid-state fermentation.

Although optimization of fermentation parameters for two- or multi-objective products in fungi has been reported, the problem of simultaneous higher production of multi-objective products has not been satisfactorily resolved. In this study, we obtained a simultaneous higher production of fruiting body and cordycepin in *C. militaris* by using a statistically based experimental design, which could have a wide application in other microbial solid-state fermentation processes. Further optimization of the cultivation environment is necessary for large-scale production of fruiting bodiesand cordycepin from *C. militaris*.

In the last few years, solid-state fermentationtechnology has been developed significantly. It has been found to be economically viable for various processes including production of pharmaceutical products. However further research is needed in the direction of automation of the process.

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