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Effects of culture conditions on mycelium biomass and intracellular cordycepin production of *Cordyceps militaris* in natural medium

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Abstract - Effects of culture conditions on the growth of mycelium and the production of intracellular cordycepin of *Cordyceps militaris* were investigated in this study. The natural medium which is a mixture of 53% brown rice paste, 6% beerwort and 42% soybean meal juice was used for the fermentation in shaking flasks. Fermentation temperature, pH and medium capacity, which had been proved to be significant factors, were optimized by Box-Behnken design. Results showed that dry mycelia weight (DMW) and cordycepin yield (CY) was varied with the transformation of culture conditions. The highest DMW (19.1 g/L) and CY (1.8 mg/g) would be obtained at the condition of fermentation temperature 28 °C, pH 6.2 and medium capacity 57 mL.

Key words: *Cordyceps militaris*; dry mycelia weight; intracellular cordycepin; culture conditions; Box-Behnken design.

INTRODUCTION

Cordyceps militaris is a kind of famous traditional medicinal mushroom in China, which belongs to the *Ascomycetes*, *Clavicepitacea, Cordyceps* and parasites on the larvae of *Lepidoptera* (Shih *et al*., 2007). It has extensively been used as tonic food and herb remedy material in China, Japan, and other Asian countries as it has many functional components which had been separated, such as cordycepin, ergosterol, adenosine and polysaccharides, etc. (Cunningham *et al*., 1950; Hubbell *et al*., 1985).

Cordycepin is a nucleoside derivative extracted from *C. militaris* (Cunningham *et al*., 1950). It performs anti-tumour, anti-bacterial, and anti-fungal functions (Kim *et al*., 2002; Zhou *et al*., 2002). It can not only inhibit the production of inflammatory mediators and the activity of adenylate cyclase in platelets (Won and Park, 2005), but also increase the intracellular levels of cAMP and cGMP in collagen-induced human platelet aggregation (Cho *et al*., 2007). In the former studies, workers had searched effective methods in order to obtain more cordycepin. And biofermentation was regarded as the optimal method, since the transformation in this course was fairly mild (Shih *et al*., 2007). Comparing the solid-state fermentation with the liquid-state fermentation, the latter was prior to the former for its shorter fermentation period.

In the liquid-state fermentation of *C. militaris*, complex organic compounds were considered as the optimum medium for the production of mycelium and cordycepin (Mao *et al*., 2005). Lee *et al*. (2004), Kim *et al*. (2005), Shih *et al*. (2007), had reported that different culture conditions had notable effects on the yield of mycelium and metabolic substance. To our knowledge, most of the fermentation of *C. militaris* used biochemical components as the culture medium, and the extracellular metabolic substances had been taken lots of attention in the researches (Mao *et al*., 2005; Masuda *et al*., 2006). However, cordycepin and other metabolic substances from mushrooms not only excrete to culture broth, but also exist in mycelia.

At the same time, it is well known that natural medium contains more nutrition for the growth of microorganisms (Lu *et al*., 2008) and the complex components in the natural medium would make it difficult to purify the metabolic substances in the culture broth. Therefore, it would be more significant to study the effects of culture conditions on the production of intracellular cordycepin in natural culture medium.

In this study, we used complex organic compounds as the medium materials, including brown rice paste, beerwort and soybean meal juice, and we attempted to elucidate the effects of culture conditions on the growth of mycelium and the production of intracellular cordycepin in natural culture medium. To our knowledge, there were few reports concerning the optimal culture conditions of intracellular cordycepin by submerged culture of *C. militaris*.

MATERIALS AND METHODS

Materials. Brown rice, malt and soybean meal were purchased from Nanjing Fair Trade Market, Jiangsu Province, China. Alpha-amylase (5000 IU/mg) was provided by Shuangxuan Microbe

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TABLE 1 - Independent variables and their levels in the Box-Behnken design

Variables	Parameter	Unit	Coded and actual level		
			-1	0	+1
X_1	Fermentation temperature	°C	22	26	30
X_2	pH		4.5	6.0	7.5
X_3	Medium capacity	ml	40	80	120

Culture Medium Products Factory in Beijing, China. Glucoamylase (10000 IU/mg) and papain (2000 IU/mg) were purchased from Wuxi Boli Bio-Products Co Ltd, and Nanjing Scigene Technology Co Ltd, Jiangsu Province, China, respectively. *Cordiceps militaris* strain was purchased from the Institute of Edible Fungus in Academy of Agricultural Science, Jiangsu Province, China.

Preparation of brown rice paste, beerwort and soybean meals juice. Brown rice was pulverized by a disintegrator (FSD-100A, Taizhou city, Zhejiang Province, China) and sifted through a 60 mesh sieve. Brown rice powder were mixed with distilled water at a ratio of 1:4 (w:v) and gelatinized at 90-95 °C for 30 min. The mixture was cooled to 60 °C, and then it was liquefied by adding α-amylase of 10000 IU/g rice powder and incubating at 60 °C for 1 h. After, the mixture was homogenized by colloid mill (DJM, Shanghai province, China) and then glucoamylase, 20000 IU/g rice powder, was added, mixed thoroughly and incubated at 60 °C up to 2 h. After maceration, the paste was sifted through a 150 mesh sieve; the soluble solid of the filtrate was adjusted to 6.0 °Brix.

Malt sprout was pulverized by a disintegrator and sifted through a 60 mesh sieve. The powder was mixed with distilled water at a ratio of 1:4 (w:v) and stayed at 35-37 °C for 30 min, at 50-55 °C for 60 min, and at 65 °C for 3 h, respectively. After maceration, the mixture was centrifuged at 4000 rpm for 10 min, its supernatant was collected and its soluble solid was adjusted to 6.0 °Brix.

Soybean meal was pulverized by a disintegrator and sifted through a 60 mesh sieve. The crushed soybean meal was mixed with distill water at a ratio of 1:10 (w:v) and boiled for 10-15 min. Then the mixture was cooled to 60 °C, after adding papain at a quantity of 4000 IU/g soybean meal powder, it was mixed thoroughly and incubated at 60 °C up to 2 h. After maceration, the mixture was centrifugated at 4000 rpm for 10 min, its supernatant was collected and its soluble solid was adjusted to 6.0 °Brix.

Maintenance and inoculum preparation. The stock culture of *C. militaris* was maintained on Potato Dextrose agar (PDA) slants and sub-cultured every month. Slants were inoculated with mycelia and incubated at 25 °C for 7 days, and then were used to prepare inoculum culture. The inoculum medium consisted of brown rice paste, beerwort and soybean meal juice (1/1/1, v/v/v). From PDA plates four discs of 6 mm in diameter were punched out and the mycelium of *C. militaris* discs was transferred to 250 mL flask containing 50 mL of the inoculum medium. The culture was incubated at 25 °C for 5 days in a reciprocating shaker at 100 rpm.

Fermentation of *Cordiceps militaris.* The natural medium was made up of 53% brown rice paste, 6% beerwort and 42% soybean meal juice, which was the optimal combination for mycelium growth and cordycepin production of *C. militaris*. The inoculation quantity was 6% (v/v), which had been determined by former studies (data not shown). After inoculum, the fermentation medium was incubated in a reciprocating shaker at 100 rpm. For optimizing the culture conditions, fermentation temperature, pH and medium capacity were designed and shown in Table 1.

TABLE 2 - Observed and predicted values of intracellular cordycepin and DMW by Box-Behnken design

Run	X_1 (°C)	X_2	X_3 (ml)	Intarcellular cordycepin (mg/g)		DMW (g/L)	
				Observed value	Predicted value	Observed value	Predicted value
1	22	4.5	80	0.82	0.72	15.71	16.08
2	30	4.5	80	1.63	1.55	15.92	15.77
3	22	7.5	80	0.97	1.05	16.72	16.42
4	30	7.5	80	0.97	1.06	16.02	16.11
5	22	6.0	40	0.34	0.33	19.82	18.93
6	30	6.0	40	1.61	1.58	18.79	18.62
7	22	6.0	120	1.19	1.22	11.15	11.97
8	30	6.0	120	0.79	0.80	11.43	11.66
9	26	4.5	40	0.44	0.55	17.26	17.68
10	26	7.5	40	1.14	1.08	19.07	19.71
11	26	4.5	120	1.15	1.21	13.03	12.40
12	26	7.5	120	0.64	0.53	11.47	11.05
13	26	6.0	80	1.68	1.65	17.56	17.87
14	26	6.0	80	1.63	1.65	17.56	17.87
15	26	6.0	80	1.73	1.65	18.44	17.87
16	26	6.0	80	1.53	1.65	17.56	17.87
17	26	6.0	80	1.68	1.65	18.21	17.87

X_1: fermentation temperature, X_2: pH, X_3: medium capacity.

Analytical method. For the measurement of dry mycelium weight (DMW), samples were centrifuged at 4000 rpm for 10 min. The mycelia were washed with distilled water and centrifuged again. Then they were transferred to pre-weighed culture dishes and dried in vacuum at 60 °C to a constant weight.

For the analysis of yield of intracellular cordycepin (YIC), 0.50 g of dried mycelia was processed ultrasonically (40 kHz 250 W) for 1 h at 50 °C with 8 mL ethanol (50%, v:v) (Wang *et al*., 2005b) . The mixture was centrifugated at 10000 rpm for 20 min. The supernatant was diluted to 10 mL with ethanol (50%, v:v), and then filtered with a 0.45 μm membrane. The filtrate was analyzed by High Performance Liquid Chromatography (HPLC) (Zhong *et al*., 2002; Masuda *et al*., 2006). Cordycepin was separated by reverse-phase HPLC using Agilent 1200 (Agilent, USA) with a Prodigy C_{18} reverse-phase column (5 μm), 4.6 x 250 mm i.d. The mobile phase was a mixture of methanol and 0.02 M potassium dihydrogenphosphate (15:85). The injection volume was 20 μL, the flow-rate was 1.0 mL/min, the working temperature was 40 °C, and the detection wavelength was 260 nm.

Experimental design. In our previous experiments, results showed that fermentation temperature, pH and medium capacity influenced the yield of mycelium and intracellular cordycepin significantly (data were not shown). Therefore, the three factors were chosen as the independent variables for the optimization by response surface methodology (RSM).

The RSM used in this study is a three-level three-factor Box-Behnken design (Table 2). The design consisted of 17 experiments, including 5 replicates. The software of Design Expert version 6.0.10 (Stat-Ease, Inc) was applied to analyze the experimental design data. In order to be correlated to the independent variables, the response variable fitted by a second order model. The general form of the second degree polynomial equation is:

$$Y = a_0 + \sum_{i=1}^{3} a_i X_i + \sum_{i=1}^{3} a_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{i<j} a_{ij} X_i X_j \qquad (1)$$

where Y is the predicted response, a_0, a_i, a_{ji}, a_{ij} are the constant coefficients, and X_i, X_j are the coded independent variables.

Statistical analysis. Analysis of variance (ANOVA) and Duncan's multiple range tests were performed in order to determine the significant difference in the production of mycelia and intracellular cordycepin by *C. militaris* under different culture conditions. A second-order polynomial regressed equation was established by analysis of Box-Behnken experimental data, and the optimum conditions for fermentation were found using the software of Design Expert version 6.0.10. All trials were carried out in triplicate and the averages were taken as responses.

RESULTS AND DISCUSSION

Dry mycelia weight and cordycepin in the fermentation

As shown in Table 3, the production of mycelia and cordycepin was increased first and decreased later with the prolonging of fermentation time. The highest yield of mycelia (15.29 g/L) and cordycepin (1.73 mg/g) was obtained at 7 days fermentation. Therefore, in our later experiment the fermentation was lasted for 7 days.

Optimization of the yield of mycelia

The RSM design for the different culture condition and fermentation results were shown in Table 2. Results showed that DMW varied considerably with the change of culture condition. Analyzing by the software of Design Expert version 6.0.10, a polynomial model (Equation 2) describing the correlation between DMW and the three variables was obtained as follows:

$$Y_{DMW} = -39.04 + 2.70X_1 + 6.20X_2 + 0.17X_3 - 0.05X_1^2 - 0.41X_2^2 - 1.08X_3^2 - 0.01X_1X_2$$
with R^2 = 0.971 (2)

The analysis of variance for Equation 2 was checked by the coefficient of determination R^2 (R^2 = 0.971), it indicated that 97.1% of the variability of DMW in the response could be explained by the model. The test statistics of P values showed that model 1 was very significant (P < 0.001). As shown in Table 4, medium capacity had significant linear and quadratic effects on the growth of *C. militaris* (P < 0.01), and the quadratic effects

TABLE 3 - Yield of mycelia and cordycepin in different fermentation time

Fermentation time (d)	DMW (g/l)	YIC (mg/g)
1	1.91 ± 0.11e	0.30 ± 0.02e
2	9.45 ± 0.65d	0.31 ± 0.03e
3	11.04 ± 0.43cd	0.75 ± 0.04d
4	13.08 ± 0.88bc	1.15 ± 0.09c
5	14.71 ± 0.37ab	1.07 ± 0.06c
6	16.08 ± 0.66a	1.45 ± 0.09b
7	15.29 ± 1.86a	1.73 ± 0.10a
8	14.00 ± 0.65ab	1.48 ± 0.02b
9	11.08 ± 1.21cd	1.14 ± 0.07c

TABLE 4 - Analysis of Variance (ANOVA) for the regression equation (2)

Source	Sum of Squares	DF	Mean Square	*F* Value	*Prob* > F
Model	121.42	7	17.35	42.67	< 0.0001
X_1	0.19	1	0.19	0.47	0.5092
X_2	0.59	1	0.59	1.46	0.2582
X_3	66.52	1	66.52	163.64	< 0.0001
X_1^2	3.00	1	3.00	7.38	0.0237
X_2^2	3.64	1	3.64	8.94	0.0152
X_3^2	12.58	1	12.58	30.95	0.0004
$X_2 X_3$	2.85	1	2.85	7.02	0.0265
Residual	3.667	9	0.41		
Lack of Fit	2.94	5	0.59	3.25	0.1384
Pure Error	0.72	4	0.18		
Cor Total	125.08	16			

X_1: fermentation temperature, X_2: pH, X_3: medium capacity.

TABLE 5 - Analysis of Variance (ANOVA) for the regression equation (3)

Source	Sum of Squares	DF	Mean Square	*F* Value	*Probability* > F
Model	3.357	9	0.373	30.049	< 0.0001
X_1	0.533	1	0.533	42.960	0.0003
X_2	0.000	1	0.000	0.002	0.9636
X_3	0.147	1	0.147	11.853	0.0108
X_1^2	0.184	1	0.184	14.859	0.0063
X_2^2	0.510	1	0.510	41.069	0.0004
X_3^2	0.898	1	0.898	72.341	< 0.0001
$X_1 X_2$	0.166	1	0.166	13.408	0.0081
$X_1 X_3$	0.697	1	0.697	56.147	0.0001
$X_2 X_3$	0.365	1	0.365	29.404	0.0010
Residual	0.087	7	0.012		
Pure Error	0.023	4	0.006		
Cor Total	3.444	16			

X_1: fermentation temperature, X_2: pH, X_3: medium capacity.

of fermentation temperature and pH were also significant (P < 0.05); the interaction effect of pH with medium capacity was significant (P < 0.05). However, both interaction effect of fermentation temperature with pH and the effect of fermentation temperature with medium capacity on the growth of *C. militaris* were not significant (P > 0.10). The above optimum parameters for the growth of *C. militaris* were evaluated by non-linear optimization algorithm and a maximum DMW of 19.69 g/L would be achieved at a temperature of 25 °C, at pH of 6.7, and a medium capacity of 50 mL.

Figure 1 shows the interaction effect of pH with medium capacity on the growth of *C. militaris*. When pH was about 6.0, medium capacity became the critical factor for the growing of *C. militaris,* DMW was reducing with the increasing of the medium capacity. When medium capacity was about 50 mL, pH became the critical factor for the growth of *C. militaris*. The DMW was found to increase at the beginning and reduce later with the elevation of pH; and when the medium capacity exceeded 75 mL, it showed a downward trend. It could be observed that the optimum pH and medium capacity for the growth of *C. militaris* were about 6.5 and 50 mL, respectively.

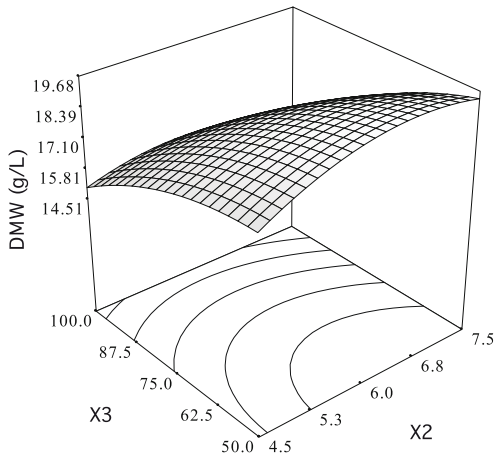

FIG. 1 - Response surface graphs for DMW of *Cordyceps militaris* as a function of pH and medium capacity.

Optimization of the production of intracellular cordycepin

By applying the software of Design Expert version 6.0.10, a polynomial model (Equation 3) describing the correlation between intracellular cordycepin production and the three variables was obtained as follows:

$$Y_{YIC} = -29.01 + 1.15X_1 + 3.12X_2 + 0.14X_3 - 0.01X_1^2 - 0.15X_2^2 - 2.89E^{-4}X_3^2 - 0.03X_1X_2 - 2.61E^{-3}X_1X_3 - 5.03E^{-3}X_2X_3 \quad (3)$$

with R^2 = 0.942

The determinate coefficient R^2 indicated the goodness of the model. Here, it is 0.942, which indicated that 94.2% of the variability in the response could be explained by the model. The statistical significance of Equation 3 was checked by *F*-test, and the analysis of variance for the fitted quadratic polynomial model is summarized in Table 5. It was found that the model is highly significant, as is evident from the model *F*-value or a very low probability value (P < 0.0001). Both fermentation temperature and medium capacity had extremely significant linear and quadratic effects on the production of intracellular cordycepin (P < 0.01); pH had extremely significant quadratic effect on it (P < 0.01). The interactions of any two of the three factors were highly significant (P < 0.01), and the relation of fermentation temperature and medium capacity was more significant than the association between pH and medium capacity. The interaction effect of fermentation temperature with pH was the least significant among them (P = 0.0081). In addition, the optimal condition for the production of intracellular cordycepin was calculated using mathematical method of extreme of multiple variables function. The optimal condition was as follows: fermentation temperature 29 ºC, pH 5.7 and medium capacity 67.4 mL. Correspondingly, the highest yield of intracellular cordycepin was 1.8 mg/g.

The fitted response surface for the production of intracellular cordycepin by the above model was generated using the Design Expert software and was given in Fig. 2 to Fig. 4.

Figure 2 showed the interaction effect of fermentation temperature with pH on the production of intracellular cordycepin. It was evident that YIC increased with the elevation of fermentation temperature, when pH was kept invariant. On the other hand, YIC enhanced gradually begin and then decreased with the increase of pH, and the turning point was 6.0. Therefore, pH 6.0 is the optimal condition. Similar results were obtained by Shih *et al*. (2007) that observed optimal production of cordycepin of *C. militaris* at pH 6.0.

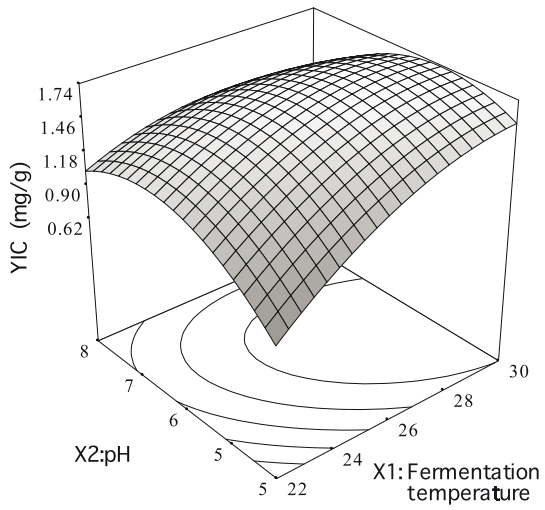

FIG. 2 - Response surface graphs for YIC of *Cordyceps militaris* as a function of pH and fermentation temperature.

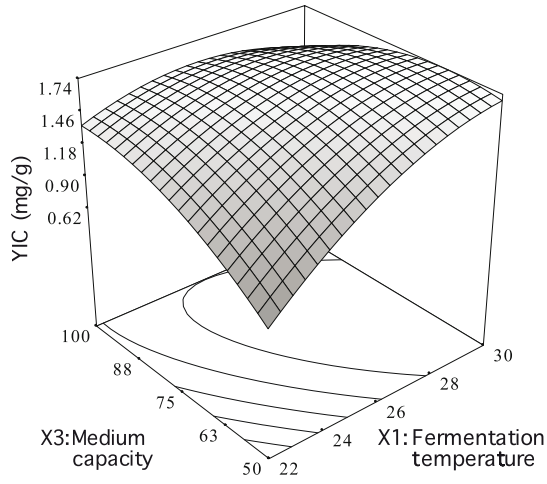

FIG. 3 - Response surface graphs for YIC of *Cordyceps militaris* as a function of medium capacity and fermentation temperature.

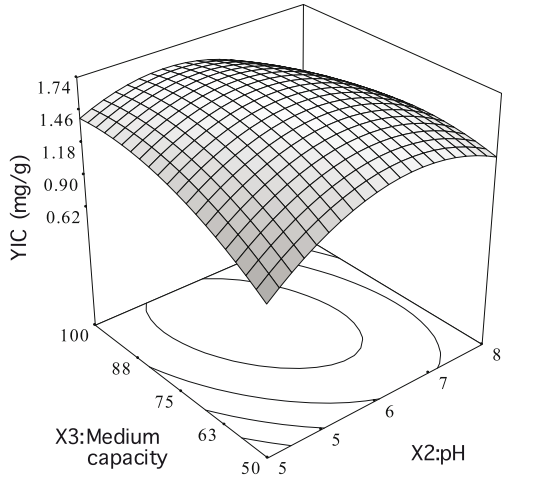

FIG. 4 - Response surface graphs for YIC of *Cordyceps militaris* as a function of medium capacity and pH.

The effect of fermentation temperature and medium capacity on the formation of cordycepin was enumerated in Figure 3. As it is shown, the yield of cordycepin raised with the increase of medium capacity when fermentation temperature was fixed, and the augment tendency became slower with the addition of medium capacity. When medium capacity was kept invariant, YIC increased with the elevation of fermentation temperature, and the scope of optimum temperature for the formation of intracellular was at about 26 to 30 °C.

Figure 4 is the plot for variation of cordycepin production, as functions of pH and medium capacity when fermentation temperature was kept at 26 °C. The yield of intracellular cordycepin was influenced by pH and medium capacity. It was observed that pH was the key factor to enhance the production of cordycepin when medium capacity was 80 mL. And the yield rose firstly and then decreased with pH increasing. The fluctuation of YIC caused by the variation of medium capacity was similar with the trend that induced by pH. Based on an overall consideration of various factors, the optimum pH and medium capacity were about 6.0 and 80 mL.

From the above analysis, the highest yield of mycelia and intracellular cordycepin could be achieved at appropriate fermentation temperature, pH and medium capacity (Fig. 1 to Fig. 4). By analyzing the plots, the optimal values of the fermentation conditions to obtain approximately 19.1 g/L DMW and 1.8 mg/g intracellular cordycepin was determined as follows: fermentation temperature 28 °C; pH 6.2 and medium capacity 57 mL.

Verification experiments

Verification experiment which was performed under the predicted culture condition by analysis of design expert 6.0 demonstrated that experimental values were generally close to the predicted values (Table 6). The result confirmed the validity and adequacy of the predicted models.

DISCUSSION

In this work, the effects of fermentation temperature, pH and medium capacity on the production of mycelium and intracellular cordycepin of *C .militaris* were studied. Importantly, the mixture of brown rice paste, beerwort and soybean meal juice was used as the fermentation medium.

As previously shown (Table 3), DMW was higher than 11.0 g/L in any fermentation conditions, which indicated that *C. militaris* grew well in the natural medium. Formerly, workers reported that *C. militaris* grew well in ample carbon and nitrogen source (Kim *et al*., 2003a, 2003b). Other researchers showed that most kinds of basidiomycete prefer to the medium of complex organic nitrogen sources in submerged fermentation (Mao and Zhong, 2006), and others reported that *C. militaris* species such as *C. militaris* C738 and *C. militaris* NG3 grew poorly in inorganic nitrogen sources (Mao *et al*., 2005; Kim *et al*. 2003a, 2003b). Brown rice, malt and soybean meal, which contain lots of complex organic substances, were regarded as food materials for human and some animals. The brown rice paste after enzymolysis contained lots of reducing sugar besides phytic acids, vitamin E and vitamin B, and so on (Standard Tables of Food Composition in Japan, 2000). Beerwort had various vitamins, such as biotin, pantothenate, thiamine, pyridoxine, riboflavin, folic acid, nicotinic acid, etc (Guan, 1997). Sometimes, soybean meal was used as nitrogen source for microorganisms (Wang *et al.*, 2005a). Therefore, the mixture of the three materials could provide sufficient nutriments for *C. militaris*.

TABLE 6 - Results of the verified experiments

Standard order*	Fermentation temperature (°C)	pH	Medium capacity (ml)	DMW (g/L)		YIC (mg/g)	
				Actual	Predicted	Actual	Predicted
1	28.0	6.2	57.0	16.68 ± 0.62	19.11	1.81 ± 0.09	1.79
2	25.0	6.0	50.0	15.84 ± 0.45	17.89	1.51 ± 0.14	1.27
3	29.5	5.7	67.4	14.98 ± 0.25	17.29	1.71 ± 0.07	1.84
4	30.0	7.5	80.0	14.13 ± 0.37	16.11	0.96 ± 0.10	1.01

* 1: optimal culture condition, 2: other's reported condition, 3: optimal condition for YIC, 4: stochastic condition.

Fermentation temperature, pH and medium capacity influenced the growth and the production of *C. militaris*, respectively. Because fermentation temperature had close relationship with the growth of mushroom fungus, it must be controlled at a certain value. Zhang (1988) reported that the befitting temperature for the growth of mushroom fungus was in the interval of 20-30 °C, and the optimum was 25 °C. In the former studies, workers always cultivated *C. militaris* at the steady temperature of 25 °C. Our results showed that *C. militaris* could grow well during 25 and 26 °C, and the production of intracellular cordycepin was elevated with the increasing of temperature in the limited scope.

pH could affect mycelial cell membrane function, cell morphology and structure, the uptake of various nutriments, and product biosynthesis (Gerlach *et al.*, 1998). The optimum values of pH for various edible mushrooms were different. Some reported that acidic condition was more suitable for the mycelial growth and the metabolites production (Park *et al.*, 2001; Hsieh *et al.*, 2005; Kim *et al.*, 2005; Shu and Lung, 2004). In this work, *C. militaris* could grow when pH was in the scope of 4.5 to 7.5, and its growth rate rose firstly with the increasing of pH, but decreased beyond pH 6.0. That was similar with the growth rhythm of *Coriolus hirsutus* (Emelyanova, 2005).

Most edible mushrooms are aerobic (Zhang, 1988) and aeration is one of the most important cultivation conditions for aerobes. In our experiments, shaking flask was used for the fermentation, so medium capacity became the important factor for obtaining dissolved oxygen. In the former studies, many have reported that oxygen supply could evidently influence the formation and accumulation of bioactive metabolites in the submerged cultivation of medicinal fungus (Zhong *et al*., 2002). Our results indicated that with the increasing of medium capacity DMW decreased but cordycepin production enhanced. Therefore, high dissolved oxygen suitable for the growth but bad for cordycepin produced.

In conclusion, the best culture condition for the growth of *C. militaris* and the production of intracellular cordycepin in natural medium was placed as follows: fermentation temperature 28 °C, pH 6.2 and medium capacity 57 mL. Under this condition, the yield of mycelium and intracellular cordycepin were 7 and 20% higher than the initial production, respectively. The information obtained in this work was helpful for the further study and the utilization of *C. militaris*.

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