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# Production of Cordycepin and Mycelia by Submerged Fermentation of *Cordyceps militaris* in Mixture Natural Culture

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**Abstract** The submerged fermentation of *Cordyceps militaris* for cordycepin production and mycelial growth was investigated in this study. Three natural materials of brown rice paste (BRP), beerwort (B), and soybean meal juice (SMJ) were used for fermentation of *C. militaris* in shaking flasks. The effects of the ratio of three natural materials on dry mycelium weight (DMW) and on cordycepin yield (CY) were analyzed. D-Optional mixture design was used to optimize the ratio of these materials. Compared with the signal culture, the higher mycelial growth and cordycepin production were obtained in mixture. The analysis of Design Expert 6.0 indicated that BRP, B, and SMJ very significantly influenced ($P<0.001$) DMW and CY of *C. militaris*, respectively. The highest DMW (18.96 g/l) and CY (2.17 mg/g) were both obtained at a ratio of 53:6:42. The experiments' results indicated that the above mixture of these natural materials by D-optional mixture design can be used as a proper medium for the growth of mycelium and the production of cordycepin.

**Keywords** *Cordyceps militaris* · Submerged cultivation · Medium · D-optimal mixture design

## Introduction

*Cordyceps militaris* is a fungus, which belongs to the class *Ascomycete*s and the group of Dong Chong Xia Cao in Chinese herbs [1]. It has extensively been used as a crude drug material and a folk tonic food in East Asia [2], as it has functional components of

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cordycepin (3-deoxyadenosine), polysaccharides, adenosine, etc. Cordycepin is a nucleoside derivative extracted from *C. militaris* [3]. It performs anti-tumor, anti-bacterial, and anti-fungal functions [4, 5]. Won and Park [6] reported that cordycepin can inhibit the production of inflammatory mediators and the activity of adenylate cyclase in platelets; then, Cho et al. [7] detected that it can also increase the intracellular levels of cAMP and cGMP in collagen-induced human platelet aggregation. Polysaccharides have anti-oxidant, anti-tumor, anti-inflammatory, and hypolipidemic effects [8, 9]. Adenosine is a cardio-protective and therapeutic agent for chronic heart failure [10]. Therefore, *C. militaris* has a potential prospect for medical application. However, this fungus has been in short supply because of its limited natural production. We have to resort to artificial cultivation of the fungus to meet the people's need, and submerged cultivation has been used as an effective method to produce *C. militaris*.

It has been reported that, in submerged cultivation of *C. militaris*, biochemical medium was used as fermentation medium [11, 12]. Some indicated that natural materials could be used as components of fungal medium [13, 14], which gives us revelation that natural materials may also be used to cultivate *C. militaris*. People generally believe in natural things more than in biochemical things. However, there has been no report on fermentation of *C. militaris* by natural medium to our knowledge. In this study, we have attempted to cultivate *C. militaris* by brown rice, malt, and soybean meal as natural materials of the culture medium, without any biochemical component.

Hydrolyzed brown rice paste (BRP) contains enough reducing sugar, which can provide a rich carbon source for the growth of *C. militaris* [15] and the formation of cordycepin [16]. Beerwort (B) was usually used as the medium of yeast. It contains some vitamins such as vitamin $B_1$, vitamin $B_2$, vitamin $B_{12}$, vitamin C, and vitamin H, apart from having plenty of carbon and nitrogen [17]. B also has lots of mineral elements such as $Ca^{2+}$, $Mg^{2+}$, and $K^+$, which may stimulate the growth of *C. militaris* and the biosynthesis of cordycepin [15]. Soybean meal was the by-product in the process of soybean oil extraction. It was always used as feed for animals, but sometimes it was used as the nitrogen source for microorganisms [18]. Hydrolyzed soybean meal juice (SMJ) can also provide nitrogen source for *C. militaris*. Now, the use of soybean meal as one component of the medium would improve its utilization value.

Therefore, the above three natural materials provide sufficient nutrients for *C. militaris*, but they must be in a proper ratio in order to achieve the highest yield of mycelium and cordycepin.

In this study, optimization of the fermentation–composition ratio of BRP, B, and SMJ was investigated by D-optimal design in order to achieve an ideal yield of mycelium and cordycepin of *C. militaris*.

## Materials and Methods

### Materials

Brown rice, malt sprout, and soybean meal were purchased from Nanjing Fair Trade Market, Jiangsu Province, China. Alpha-amylase (5,000 IU/mg) was provided by Shuangxuan Microbe Culture Medium Products Factory in Beijing, China. Glucoamylase (10,000 IU/mg), papain (2,000 IU/mg), and *C. militaris* strain were purchased from Wuxi Boli Bio-Products Co Ltd, Nanjing Scigene Technology Co Ltd, and the Institute of Edible Fungus in Academy of Agricultural Science, Jiangsu Province, China, respectively.

## Maintenance and Seed Culture of *C. militaris*

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 used for seed culture inoculation. The seed culture medium consisted of BRP, B, and SMJ (*v*/*v*/*v*, 1:1:1). The mycelia of *C. militaris* were transferred to the seed culture medium by punching out agar discs of 6 mm in diameter on PDA plates. Four discs were inoculated into a 250-ml shaking flask, which contains 50 ml culture medium, and then they were incubated at 25 °C on a reciprocating shaker (100 rpm) for 5 days.

## Preparation of BRP, B, and SMJ

Brown rice was pulverized by a disintegrator (FSD-100A, Taizhou City, Zhejiang Province, China) and sifted through a 60-mesh sieve. Brown rice powder was 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 $\alpha$-amylase of 10,000 IU/g rice powder and incubating at 60 °C for 1 h. After liquefaction, the mixture was homogenized by colloid mill (DJM, Shanghai Province, China) and then added with glucoamylase at a quantity of 20,000 IU/g rice powder, mixed thoroughly, and incubated up to 2 h at 60 °C. 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 4,000 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 distilled 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 4,000 IU/g soybean meal powder, it was mixed thoroughly and incubated up to 2 h at 60 °C. After maceration, the mixture was centrifuged at 4,000 rpm for 10 min. Its supernatant was collected, and its soluble solid was adjusted to 6.0 °Brix.

The actual values of the major components in the above materials were analyzed.

## Fermentation Conditions

Single and mixture of BRP, B, and SMJ was fermented of *C. militaris*, respectively. Optimization of the ratio of the mixture was studied, and the composition of medium in this experiment was shown in Table 1. The fermentations were carried out in 250-ml flasks with 50 ml culture medium. The fermented medium was inoculated with 10% (*v*/*v*) of the seed culture and then cultivated for 7 days at 25 °C; its pH and rotating times were controlled at 6 and 100 rpm, respectively.

## Analytical Methods

For the measurement of DMW, samples were centrifuged at 4,000 rpm for 10 min. The substrates were washed with distilled water and centrifuged again. Then, they were transferred onto pre-weighted culture dishes and dried at 60 °C to a constant weight.

For the analysis of cordycepin, 0.50 g of dried mycelia was sonicated (20 kHz, 250 W) for 1 h at 50 °C with 8 ml ethanol (50%, *v*/*v*) [19]. Working frequency was fixed at 40 kHz.

**Table 1** Values of DMW and CY by D-optimal mixture design.

| Standard order | $X_1$ (%) | $X_2$ (%) | $X_3$ (%) | DMW (g/l) | CY (mg/g) |
|---|---|---|---|---|---|
| 1 | 40 | 5 | 55 | 15.39±0.60 | 1.63±0.03 |
| 2 | 40 | 30 | 30 | 12.99±0.74 | 0.55±0.01 |
| 3 | 65 | 5 | 30 | 16.23±1.23 | 1.66±0.04 |
| 4 | 40 | 55 | 5 | 9.95±0.32 | 0.56±0.02 |
| 5 | 65 | 30 | 5 | 8.74±0.18 | 0.47±0.02 |
| 6 | 90 | 5 | 5 | 9.34±0.05 | 0.29±0.01 |
| 7 | 48.33 | 13.33 | 38.33 | 17.19±0.09 | 1.40±0.06 |
| 8 | 57.67 | 22.67 | 22.67 | 13.18±1.79 | 0.49±0.02 |
| 9 | 48.33 | 38.33 | 13.33 | 9.66±1.12 | 0.26±0.03 |
| 10 | 73.33 | 13.33 | 13.33 | 10.14±2.23 | 0.37±0.02 |
| 11 | 40 | 5 | 55 | 15.39±0.60 | 1.63±0.03 |
| 12 | 40 | 55 | 5 | 9.95±0.33 | 0.56±0.02 |
| 13 | 90 | 5 | 5 | 9.30±0.12 | 0.37±0.02 |
| 14 | 65 | 30 | 5 | 8.74±0.29 | 0.47±0.02 |

$X_1$=BRP, $X_2$=B, $X_3$=SMJ

The mixture was centrifuged at 10,000 rpm for 20 min. The supernatant was diluted to 10 ml with ethanol (50%, *v*/*v*) and then filtered with a 0.45 membrane. The filtrate was analyzed by high-performance liquid chromatography (HPLC) [16, 20, 21]. Cordycepin was separated by reverse-phase HPLC using Agilent 1200 (Agilent, USA) with a Prodigy $C_{18}$ reversed-phase column (5 μm), 4.6×250 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.

The content of reducing sugar was assayed by applying 3,5-dinitrosalicylic acid method [22], the concentration of protein was measured by colorimetric method with Coomassie Brilliant Blue G-250 as colorimetric liquid, and the free amino nitrogen concentration was assayed using the ninhydrin colorimetric method [23].

## D-optimal Mixture Design

Mixture experiment is a special type of response surface experiment in which the factors are the components of a mixture, and the response is a function of the proportions of each ingredient. The mixture components cannot range in an independent way since their sum has to be equal to 1, and specific experimental matrices and mathematic models have to be used [24]. This approach has been used not only to solve pharmaceutical blending problems but also for optimizing mixture composition in engineering, in food industry, and in compost formulation [25, 26].

In this study, D-optimal mixture design was conducted in the optimum vicinity to locate the true optimum ratio of BRP ($X_1$), B ($X_2$), and SMJ ($X_3$) for *C. militaris* growth and cordycepin production. According to statistic theory, a D-optimal mixture design of three factors consisted of 14 experiments, including six model points, four estimated lack of fit, and four replicates (Table 1). The design space is defined by the low and high level constraints on each factor and any multifactor constraints [27]. The usual mixture restraints apply to the proportion of variables with $X_1 + X_2 + X_3 = 1$ [28]; the proportion of the three materials was $0.4 \leq X_1 \leq 0.9,\ 0.05 \leq X_2 \leq 0.55,$ and $0.05 \leq X_3 \leq 0.55$, respectively. Through experiments, the regression equation about these three materials with DMW and with CY was built, respectively. The experimental results were fitted with the Scheffe third-order polynomial equation of Eq. 1.

$$Y = \sum_{j=1}^{n} b_j X_j + \sum_{h<j} b_{hj} X_h X_j + \sum_{h<j<k} b_{hjk} X_h X_j X_k \quad \begin{cases} X_j \geq 0 \\ \sum_{j=1}^{n} X_j = 1 \end{cases} \left(j = {}^{1,2,3,\cdots n}\right) \tag{1}$$

where $Y$ is predicted response, $b_j$, $b_{hj}$, and $b_{hjk}$ are the regression coefficient for linear, quadratic, and special cubic mixture models, respectively, and $X_j$, $X_h$, and $X_k$ are the coded independent variables; the $X$ variables represent their proportions in this design, where $n$ equals to the number of the tested factors and now is 3. The third-order polynomial equation in this text is presented as follows:

$$Y = b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{123}X_1X_2X_3 \tag{2}$$

### Statistical Analysis

Conventional statistical methods were used to calculate the means and standard deviations of the experiment. Analysis of variables (ANOVA) was applied to determine significant differences at $P < 0.05$. Contrasts (least significant difference test) between means were made to examine the level of the difference between the variable factors. One-way ANOVA was done using Statistica for Windows Release 5.0A (Statsoft Inc., Tulsa, OK, USA). Three independent experiments were carried out in this study.

## Results and Discussions

### Main Nutritional Components of the Different Materials

The main nutritional components in BRP, B, and SMJ were analyzed in this study (Table 2). The results showed that SMJ contained the most soluble protein and free amino acids, which indicated that SMJ provided the nitrogen source for *C. militaris* growth and cordycepin production. Both BRP and B contained lots of reducing sugar; they would offer the carbon source for *C. militaris*.

**Table 2** Main nutritional components and related indexes of different culture medium.

| Material | Indexes | | |
|---|---|---|---|
| | Reducing sugar (g/l) | Soluble protein (mg/l) | Free amino acids (mg/l) |
| BRP | 74.28±1.00b | 328.83±27.66b | 120.27±4.12c |
| B | 105.93±1.36a | 310.64±27.52b | 207.58±8.80b |
| SMJ | 1.94±0.02c | 2225.57±40.18a | 1071.33±28.02a |

a, b, c = the significant difference of the experiment results at $P<0.05$

## Fermentation of *C. militaris* by BRP, B, SMJ and Their Mixture

The single and mixture of BRP, B, and SMJ were used for fermentation of *C. militaris* in this experiment (Table 3). The result indicated that *C. militaris* grew well both in single and mixture of the three materials. The highest DMW (14.70 g/l) and CY (0.66 mg/g) were obtained at their mixture; that is because the mixture gave a comprehensive nutrition for the mycelial growth and cordycepin production of *C. militaris.* The higher DMW (11.09 g/l) was obtained at B for B contains carbon and nitrogen source (Table 2). However, the higher CY (0.49 mg/g) was obtained at SMJ. This may be due to the substrate or precursor of enzyme, which was vital for the creation of cordycepin in SMJ; however, this needs further research. Due to lack of enough nitrogen, the least yield of mycelium (6.77 g/l) and cordycepin (0.26 mg/g) was obtained at BRP.

In a word, the mixture of the three materials gave more sufficient nutrition for *C. militaris*. The effect of the material ratios on the yield of mycelium and cordycepin was investigated in the experiments discussed below.

## Optimization of the Material Ratio

### *Fitting the Model*

The D-optimal designs for different ratios of the materials and fermentation results were shown in Table 1. By applying Design Expert 6.0 analysis, the experimental results were fitted with the Scheffe third-order polynomial equation of Eq. 2, and the Scheffe third-order polynomial equations for DMW ($Y_{\mathrm{DMW}}$) and for CY ($Y_{\mathrm{CY}}$) were shown in Eqs. 3 and 4, respectively. Their regression analyses were shown in Tables 4 and 5, respectively.

$$\text{Model 1 } Y_{\mathrm{DMW}} = 5.98X_1 + 10.77X_2 - 1.81X_3 + 63.31X_1X_3 \tag{3}$$

$$Y_{\mathrm{CY}} = -0.37X_1 - 0.64X_2 - 2.80X_3 + 4.91X_1X_2 + 15.99X_1X_3 + 31.01X_2X_3 - 97.23X_1X_2X_3 \tag{4}$$

The ANOVA for Eq. 3 was checked by the coefficient of determination $R^2$ ($R^2$=0.9230); it indicated that 92.30% of the variability of DMW in the response could be explained by model 1. The test statistics of *P* values showed that model 1 was very significant ($P<$ 0.001). As shown in Table 4, both the linear mixture and the interaction of BRP with SMJ effect on the growth of *C. militaris* were significant ($P<0.05$). However, the interactive effects of BRP with B and B with SMJ on the growth of *C. militaris* were not significant ($P>0.10$).

The analysis of variance for Eq. 4 was checked by the coefficient of determination $R^2$ ($R^2$=0.9609), which indicated that 96.09% of the variability of CY in the response could be

**Table 3** MValues of DMW and CY of *C. militaris* by single and mixture of BRP, B and SMJ.

| Index | BRP | B | SMJ | BRP/B/SMJ 1:1:1 |
|---|---|---|---|---|
| DMW (g/l) | 6.77±0.83d | 11.09±0.55b | 8.08±0.06c | 14.70±0.11a |
| CY (mg/g) | 0.26±0.05d | 0.32±0.03c | 0.49±0.04b | 0.66±0.07a |

a, b, c = the significant difference of the experiment results at $P<0.05$

**Table 4** Analysis of variance for DMW with the regression model.

| Source | Sum of squares | *df* | Mean square | *F* value | Probability>F |
|---|---|---|---|---|---|
| Model | 113.45 | 3 | 37.82 | 39.95 | 0.0001 |
| Linear mixture | 88.09 | 2 | 48.54 | 51.29 | <0.0001 |
| $X_1X_3$ | 16.75 | 1 | 16.36 | 17.28 | 0.0020 |
| Residual | 9.47 | 10 | 0.95 | | |
| Corrected total | 113.9 | 13 | | | |

Statistical experimental design: $R^2$ =0.9230; three factors, 14 runs; $X_1$=BRP, $X_2$=B, $X_3$=SMJ

explained by model 2. The test statistics of *P* values shows that the model, the linear mixture, and the interaction between BRP and SMJ were very significant ($P< 0.01$; Table 5). The interactions between B and SMJ and between the three materials on the influence of cordycepin were significant, respectively ($P<0.05$). However, the interaction of BRP with B was not significant ($P>0.10$).

### *Contour Plots and Response Surface Analysis*

Two-dimensional contour plots and dimensional response triangular surface, as presented in Figs. 1 and 2, are very useful to observe the effects of the factors on the responses.

As shown in Fig. 1, a low DMW was obtained at a high accession of B because B contained lots of maltose, which was not the optimum carbon source for the growth of *C. militaris* [16]. However, when the ratio of B was lower than about 5%, DMW decreased; a lower ratio of B means lower vitamin and mineral element contents, which may stimulate the growth of *C. militaris* and the biosynthesis of cordycepin [15]. DMW was enhanced by the proper increase in the ratio of SMJ, but it declined when this ratio was over 43%. In a certain range (50–90%), the increase in BRP led to the reduction in DMW. The change of the material ratios leads to the variation of carbon–nitrogen ratio, which influenced the growth of *C. militaris* [29]. The highest yield (18.96 g/l) of DMW was reached when the ratio of the three materials is as follows: 52% BRP, 43% SMJ, and 5% B. At this time, the mixture contains 44.75 g/l reducing sugar, 1,143.52 mg/l soluble protein, and 533.59 mg/l free amino acids.

It was evident from Fig. 2 that CY varied from 0.26 to 1.63 mg/g in the level constraints and was significantly affected ($P<0.01$) by the ratio of the above three materials. The

**Table 5** Analysis of variance for CY with the regression model.

| Source | Sum of squares | *df* | Mean square | *F* value | Probability>F |
|---|---|---|---|---|---|
| Model | 3.74 | 6 | 0.62 | 28.47 | 0.0001 |
| Linear mixture | 2.88 | 2 | 1.44 | 65.88 | <0.0001 |
| $X_1X_2$ | 1.22E–05 | 1 | 1.22E–05 | 5.57E–04 | 0.9818 |
| $X_1X_3$ | 0.40 | 1 | 0.40 | 18.40 | 0.0036 |
| $X_2X_3$ | 0.20 | 1 | 0.20 | 9.23 | 0.0189 |
| $X_1X_2X_3$ | 0.16 | 1 | 0.16 | 7.31 | 0.0305 |
| Residual | 0.15 | 7 | 0.022 | | |
| Corrected total | 3.89 | 13 | | | |

Statistical experimental design: $R^2$ =0.9606; three factors, 14 runs; $X_1$=BRP, $X_2$=B, $X_3$=SMJ

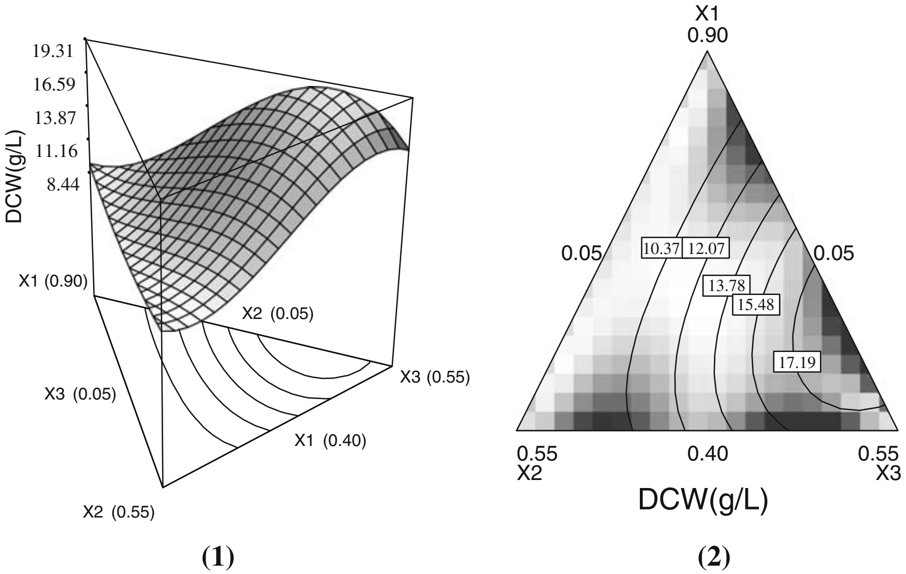


**Fig. 1** Response triangular surface (*1*) and contour plot (*2*) for dry mycelium weight (DMW) to BRP ($X_1$), B ($X_2$), and SMJ ($X_3$)

change in trend of CY was similar with DMW on the variation of the ratios of the three materials. Only the inflection point of the ratio was a little different. In a word, CY changed with variation of the ratio of these materials because the concentrations of carbon and nitrogen as well as their ratios influenced the production of cordycepin [16]. The higher

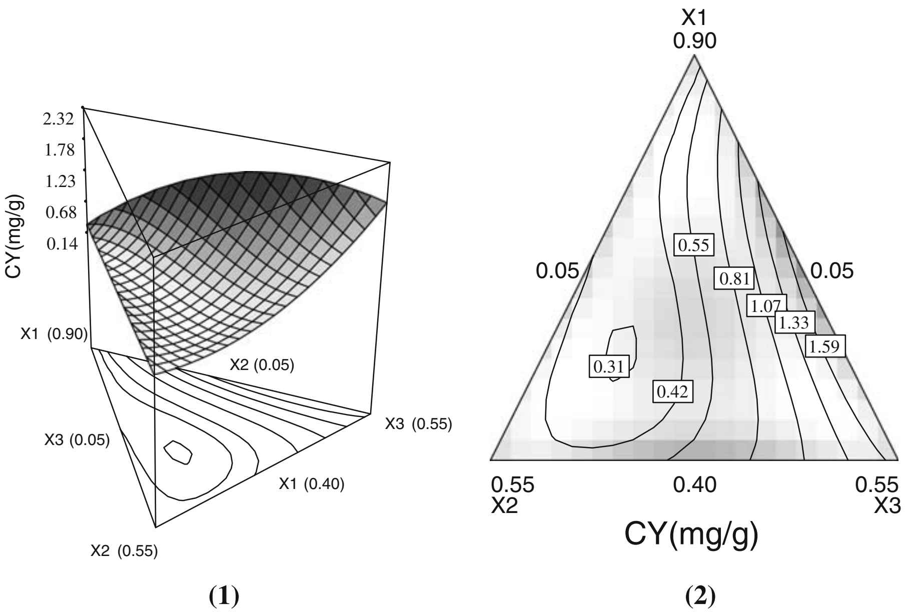


**Fig. 2** Response triangular surface (*1*) and contour plot (*2*) for cordycepin yield (CY) to BRP ($X_1$), B ($X_2$), and SMJ ($X_3$)

yield (2.17 mg/g) of cordycepin was reached at 51% BRP, 5% B, and 44% SMJ, which was the optimum ratio for the creation of cordycepin. In the optimal mixture, its content of main nutrition was similar with the optimal mixture for the mycelial growth of *C. militaris*.

A positive correlation was found between DMW and CY ($r$=0.87), indicating that a higher DMW was related to a higher CY. The model predicted a maximum yield of 18.96 g/l DMW, and 2.17 mg/g cordycepin was reached at 53% BRP, 6% B, and 42% SMJ.

## Conclusion

The present study indicated that *C. militaris* grew well in the natural medium, BRP, B, and SMJ. Through the D-optimal design, higher DMW (18.96 g/l) and cordycepin yield (2.17 mg/g) were obtained at a ratio of 53% BRP, 6% B, and 42% SMJ. The experimental results obtained in this work confirmed the previous supposition that *C. militaris* could be cultivated by natural materials. It is useful to ferment *C. militaris* by natural medium to obtain higher yield of mycelium and cordycepin.

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