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Analysis of appearance and active substances of *Cordyceps militaris* stromata on *Antheraea pernyi* pupae after optimization

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Abstract

Cordyceps militaris stromata on *Antheraea pernyi* pupae contain various active components, including cordycepin, adenosine, polysaccharides, and amino acids. Response surface methodology (RSM) was used to optimize the liquid culture conditions for *C. militaris* before its injection into *A. pernyi* pupae. A pH of 7.56 ± 0.02, a culture temperature of 20.5 ± 0.1 °C, a culture time of 110.5 ± 0.5 h, and a KH_2PO_4 concentration of 1.11 ± 0.01 g l^{-1} resulted in a *C. militaris* dry weight of 1.0226 g l^{-1}. Experimental and predicted values were similar. The RSM optimization increased the number of fruiting bodies (17 to 22) and the average fruiting body length (6.9 cm to 7.9 cm), while also deepening the yellow colouration of the fruiting bodies. The adenosine, cordycepin, polysaccharide, carotenoid, and cordycepic acid contents increased by 12.52%, 7.67%, 3.03%, 14.93%, and 0.02%, respectively, after the optimization. However, the optimization did not alter the number of different amino acids (18) or the total amino acid content, even though the contents of certain amino acids changed somewhat. These finding may be useful for increasing the yield of *C. militaris* stromata on *A. pernyi* pupae, which will increase the profitability of *C. militaris* production.

Keywords: *Cordyceps militaris*; response surface methodology; cordycepin; adenosine; polysaccharides; amino acids.

Practical Application: In this study, after the RSM optimization, the average fruiting body length increased from 6.9 cm to 7.9 cm and the colour changed from light yellow to dark yellow. The adenosine, cordycepin, polysaccharide, carotenoid, and cordycepic acid contents increased by 12.52%, 7.67%, 3.03%, 14.93%, and 0.02%, respectively, following the optimization. Some amino acid contents also changed to varying degrees. The study findings will help to increase the quality of *C. militaris* stromata on *A. pernyi* pupae, thereby increasing the economic value of *C. militaris*.

1 Introduction

Cordyceps militaris, which is an edible medicinal fungus distributed worldwide (Chen et al., 2021), is one of more than 700 species of entomopathogenic fungi that have been identified (Fan et al., 2021b); these species include 300 that produce fruiting bodies. Two entomopathogenic fungi are known to be widely applied (Lee et al., 2017), namely *Cordyceps sinensis* in the Qinghai-Tibetan Plateau (QTP) and *C. militaris*. However, *C. sinensis* can only parasitize ghost moth (*Thitarodes* spp.) larvae (Lin et al., 2017), which is distributed on the Tibetan Plateau at high altitudes of between 3600 and 4800 m (Yin et al., 2017), this fungus exhibits extremely high host specificity. The strict environmental conditions required for growth and the fact it has only one host have limited the yield of *C. sinensis*. In contrast, *C. militaris*, which can grow under relatively broad environmental conditions, may be cultured on diverse substrates, including grains (e.g., wheat) and *Antheraea pernyi* pupae (Figure 1) (Miao et al., 2020; Choi et al., 2021). Because of the differences in the substrates, *C. militaris* stromata on grains may be distinguished from *C. militaris* stromata on *A. pernyi* pupae (Quan et al., 2020). When *C. militaris* is cultivated on grains (e.g., wheat), the generated fruiting bodies are used (Jo et al., 2020). Only a small amount of the culture medium is used as a feed additive for animal husbandry (Raethong et al., 2018), with the remaining culture medium treated as industrial waste (Nallathamby et al., 2020), which pollutes the environment and wastes resources. If *C. militaris* is cultivated on *A. pernyi* pupae, the culture substrate and the fruiting bodies are used and the formation of by-products is limited (Li et al., 2021). A recent study showed that *C. militaris* stromata on *A. pernyi* pupae are superior to *C. sinensis* in terms of their active compounds, such as cordycepin (Liu et al., 2021). Because of its characteristics of high quality and low by-product, cultivating *C. militaris* stromata on *A. pernyi* pupae may be a high-performance production scheme.

Cordyceps militaris is primarily produced in China. The annual output of *C. militaris* in China is now worth more than 10 billion RMB. The adenosine, protein (Soltani et al., 2017), and essential amino acid contents are reportedly higher when the fungus is cultured on *A. pernyi* pupae than when it is cultured on grains (Meng et al., 2020). Additionally, *C. militaris* stromata cultured on *A. pernyi* pupae can enhance immunity and prevent cancer, oxidative stress-induced damages, and cardiovascular and cerebrovascular diseases. *Antheraea pernyi* pupae are useful for cultivating *C. militaris* stromata because they contain important nutritional components. Although the cost of this cultivation is relatively high, the resulting stromata may be sold at prices that are up to 10-times higher than the price of *C. militaris* stromata

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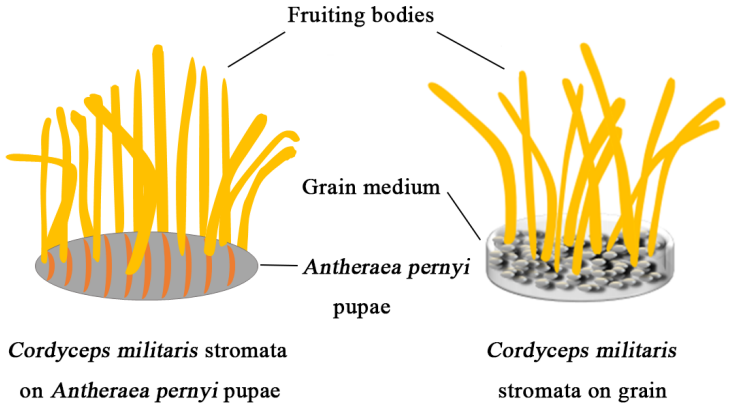

Figure 1. Differences between *C. militaris* stromata on *A. pernyi* pupae and *C. militaris* stromata on grains.

derived from grains (Rupa et al., 2020). Therefore, methods for increasing the yield of *C. militaris* stromata on *A. pernyi* pupae should be developed. The process for cultivating *C. militaris* stromata on *A. pernyi* pupae is similar to that for *C. sinensis* stromata (Fan et al., 2021a), with both fungi producing similar active ingredients (Lee et al., 2020). Thus, *C. militaris* stromata on *A. pernyi* pupae are a good substitute for *C. sinensis*. There is currently an increasing demand for *C. militaris* stromata cultivated on *A. pernyi* pupae in China, but low yields have seriously affected their mass production and application, thereby limiting the economic benefits of *C. militaris* production (Zou et al., 2022). Accordingly, the objective of this study was to provide researchers with relevant data for increasing the yield and quality of *C. militaris* stromata on *A. pernyi* pupae on the basis of a series of experiments.

There are two main methods to increase the active substance compounds in *C. militaris*, such as improving the culture conditions and developing stress-tolerant strains. For example, Wang et al. (2021) more than doubled cordycepin production using hypoxia-tolerant *C. militaris*. Response surface methodology (RSM) has often been used to optimize *C. militaris* growth conditions. To the best of our knowledge, this is the first study to attempt to apply RSM to optimize the culture conditions that is then injected into *A. pernyi* pupae to increase the contents of active compounds in *C. militaris* stromata on *A. pernyi* pupae. The data presented herein may be relevant for modulating the production of *C. militaris* stromata on *A. pernyi* pupae to increase the yield of active substances. Some studies have shown that the pre-inoculation *C. militaris* concentration has critical effects on the growth of fruiting bodies, which is directly related to the yield, quality, and economic benefits of *C. militaris* (Song et al., 2021; Prommaban et al., 2022). Unfortunately, there has been limited research on the cultivation of *C. militaris* stromata on *A. pernyi* pupae or the *C. militaris* concentration before its injection into *A. pernyi* pupae.

In this study, the *C. militaris* dry weight was used as an index and experiments were designed to investigate the effects of the following four factors: culture temperature, pH, KH_2PO_4 concentration, and culture time, each at three levels. The results were used to determine the optimal liquid culture conditions for producing *C. militaris* before it is injected into *A. pernyi* pupae. The growth and colour of the fruiting bodies on *C. militaris* stromata on *A. pernyi* pupae should be improved by optimizing the liquid culture conditions to enhance the production of active substances.

2 Materials and methods

2.1 Materials

Cordyceps militaris (L.) Fr. was purchased from the Institute of Microbiology of the Chinese Academy of Sciences, Beijing, China. *Antheraea pernyi* Guérin-Méneville (Lepidoptera: Saturniidae) pupae were provided by the Sericultural Research Institute of Jilin Province, Jilin, China. The *A. pernyi* pupae were 35.0 ± 0.2 mm long and 19.0 ± 0.2 mm wide, with a fresh weight of 9.0 ± 0.1 g. The potato dextrose agar (PDA) medium, ethanol, glucose, phenol, and sulfuric acid were analytically pure and purchased from the National Medicine Group Chemical Reagent Co., Ltd. (Shanghai, China). Chromatographically pure cordycepin, adenosine, and cordycepic acid were purchased from Fisher Clinical Services (Allentown, PA, USA). Amino acid standards were purchased from Sykam (Amino Acid Calibration Solution H; Sykam, Germany). Carotenoid was purchased from Tokyo Chemical Industry Co., Ltd. All other chemicals were of analytical grade. The pH was measured using a digital pH meter (pH 211 Microprocessor pH Meter; Hanna Instruments, Ann Arbor, MI, USA).

2.2 Response surface methodology optimization of Cordyceps militaris growth before the injection into Antheraea pernyi pupae

Cordyceps militaris was cultured on PDA solid medium (pH 7) at 21 °C. An agar plug (about 0.5 cm^2) containing *C. militaris* was used to inoculate 100 mL PDA liquid medium (pH 7). The mycelia were grown in the PDA liquid medium at 21 °C (150 rpm) in a shaking incubator (Thermoshaker PST-60HL-4; Lab4You, Berlin, Germany) until the optical density at 600 nm was 0.5-0.6. Next, 1-mL aliquots of the mycelial cultures were used to inoculate fresh liquid medium (pH 6, 7, and 8) containing KH_2PO_4 (0.5, 1.0, and 1.5 g l^{-1}) for culturing at 19, 21, and 23 °C (150 rpm) in a shaking incubator for 72, 96, and 120 h. The culture medium was centrifuged at 5,000 g for 10 min

(Waltham, MA, USA) and the supernatant was discarded to obtain pure mycelia. The collected mycelia were transferred to dry filter paper and dried at 40 ± 2 °C to a constant weight. The weight of the dried mycelia was recorded using the ARA520 electronic balance (JP-3000WP; Chyo Balance Co., Kyoto, Japan).

The Design-Expert 8.06 software (Stat-Ease Inc., Minneapolis, MN, USA) was used for designing experiments, analysing data, and validating results. The Box–Behnken design was used for the RSM optimization. The data analysis was performed using the dry weight of the *C. militaris* mycelia grown in liquid medium as the index. Experiments, which were completed in triplicate, were designed to examine the effects of four factors (pH, culture temperature, KH_2PO_4 concentration, and culture time), with three levels per factor (Table S1).

2.3 Cultivation of Cordyceps militaris stromata on Antheraea pernyi pupae

Cordyceps militaris was cultured on PDA solid medium (pH 7) at 21 °C. An agar plug (about 0.5 cm^2) containing *C. militaris* was used to inoculate 100 mL PDA liquid medium (pH 7). In this study, two methods were used to prepare *C. militaris* suspensions (i.e., before and after the RSM optimization). Before the optimization, mycelia were grown in PDA liquid medium (pH 7) containing 1.0 g l^{-1} KH_2PO_4 at 21 °C (150 rpm) for 100 h in a shaking incubator (Thermoshaker PST-60HL-4). After the optimization, mycelia were grown in PDA liquid medium (pH 7-8) containing 1.11 g l^{-1} KH_2PO_4 at 20-21 °C for 110-111 h. The *C. militaris* mycelial suspension was obtained and injected into individual *A. pernyi* pupae (0.2 mL injection volume), which were subsequently incubated at 21 °C and 65% relative humidity under incandescent light (200 lx) (Shuangyi Lighting Electric Appliance Co., Ltd., Shenzhen, China) for 30 days.

2.4 Determination of the adenosine and cordycepin contents

Cordyceps militaris stromata on *A. pernyi* pupae were collected and dried at 40 °C in the DHG-9123A oven (Shanghai Lang Gan Experimental Equipment Co., Ltd.) to a constant weight. The dried material was pulverized and then filtered through a 100-mesh sieve. Next, 0.5 g *C. militaris* powdered material was added to 100 mL distilled water (final volume). After a thorough shaking using the T25 homogenizer (Staufen, Germany), the suspension was extracted using an ultrasonicator (Kun Shan Ultrasound Instrument Co., Jiangsu, China) at 20 °C for 3 h and then centrifuged at 5,000 g for 15 min. The supernatant was collected and passed through a 0.45-μm syringe filter (Whatman Inc., Maidstone, UK). The active substance contents in the filtrate were measured by high-performance liquid chromatography (HPLC) (Singpoonga et al., 2020).

Adenosine and cordycepin were precisely weighed and then formulated to produce standard solutions with a concentration range of 0.1-20 μg mL^{-1}. Samples (10 μL) were injected into the Accela HPLC system (Thermo Scientific, Bellefonte, PA, USA) for three parallel analyses, with each analysis repeated three times. The HPLC conditions were as follows: Ultimate XB C18 column (5 μm, 4.6 mm × 250 mm; Welch Materials Inc., West Haven, CT, USA); mobile phase, water:acetonitrile at 90:10 (v/v); flow rate, 1 mL min^{-1}; column temperature, 30 °C; injection volume, 10 μL; and detection wavelength, 260 nm.

The peak areas of the adenosine and cordycepin standards at different concentrations were separately recorded in triplicate and the average values were calculated. The peak areas of the different concentrations of a mixed standard solution were determined by HPLC and then a standard curve was prepared using the peak area as the ordinate and the mixed standard solution concentration as the abscissa. The linear equations of the standard calibration curves for adenosine and cordycepin were respectively $y = 0.8855x + 0.0111$ ($R^2 = 0.999$) and $y = 0.6133x - 0.023$ ($R^2 = 0.999$).

2.5 Determination of the polysaccharide content

The polysaccharide content was determined using the phenol–sulfuric acid method, with glucose as the standard (Yuan et al., 2018). A standard curve was drawn using the optical density value as the ordinate and the standard concentration as the abscissa. The linear equation for the standard calibration curve was $y = 15.793x - 0.0013$ ($R^2 = 0.999$).

2.6 Determination of the carotenoid content

Cordyceps militaris stromata on *A. pernyi* pupae were dried at 40 °C for 48 h and filtered through a 30-mesh sieve to obtain the sample powder. A certain amount of the sample was weighed and mixed with 60% ethanol (i.e., extraction agent) for a solid:liquid ratio of 1:20 (w/v). After an overnight extraction at 25 °C, the sample was centrifuged at 8,000 rcf for 10 min and the supernatant was collected as the crude carotenoid extract. The absorbance was measured at 470 nm using the U-3010 UV-Vis spectrophotometer (Hitachi, Tokyo, Japan) (Zhang et al., 2018). The carotenoid content was calculated using the following Equation 1:

$$carotenoid\ content\left(g\ kg^{-1}\right) = \frac{A \times V \times D}{0.16W \times 1,000} \quad (1)$$

where A is the absorbance, V is the volume of 60% ethanol (mL), D is the dilution co-number of the extract, 0.16 is the extinction coefficient, W is the sample dry weight (g), and 1,000 is the conversion factor.

2.7 Determination of the cordycepic acid content

Cordyceps militaris powdered material (0.5 g) was added to 100 mL distilled water (final volume). After a thorough shaking using the T25 homogenizer, the suspension was extracted using an ultrasonicator (Kun Shan Ultrasound Instrument Co.) at 40 °C for 3 h and then centrifuged at 5,000 g for 15 min. The supernatant was collected and passed through a 0.45-μm syringe filter (Whatman Inc.). The active substance contents in the filtrate were measured by HPLC.

Cordycepic acid was precisely weighed and then formulated to produce standard solutions with a concentration range of 0.5-50 μg mL^{-1}. The HPLC injection volume was 10 μL and three parallel analyses were conducted for each sample, with each analysis repeated three times. The HPLC conditions were as follows:

Ultimate XB-C18-column (5 μm, 4.6 mm × 250 mm; Welch Materials Inc.); mobile phase, water: acetonitrile at 22:78 (v/v); flow rate, 1.0 mL min^{-1}; column temperature, 35 °C; injection volume, 10 μL; and detection wavelength, 290 nm. The linear equation for the standard calibration curve for cordycepic acid was y = 0.7007x − 0.1868 (R^2 = 0.999).

2.8 Determination of the amino acid content

After transferring 30 mg *C. militaris* powdered material to a hydrolysis tube, 6 mL 6 mol l^{-1} hydrochloric acid, 10 μL phenol, and nitrogen were added. The hydrolysis tube was sealed and incubated at 110 °C for 22 h. The hydrolysate was transferred to a 25-mL volumetric flask containing pure water and the volume was adjusted to the calibration line. A 1-mL aliquot of the sample solution was added to a 10-mL centrifuge tube, which was placed in an oven to dry the sample at 40 °C under vacuum conditions. The residue was dissolved with 1 mL water and dried twice before it was finally dissolved in 1 mL 0.2 mol l^{-1} HCl solution and passed through a 0.22-μm microporous membrane. The amino acid composition was analysed using the H835-50 automated amino acid analyser (Hitachi). The amino acid standards were processed as described above.

3 Results and Discussion

3.1 Regression analysis

The process for culturing *C. militaris* stromata on *A. pernyi* pupae is complex. The growth of *C. militaris* before it is injected into *A. pernyi* pupae is crucial for the development of *C. militaris* stromata on *A. pernyi* pupae. The Box–Behnken design was applied to optimize selected variables (pH, culture temperature, culture time, and KH_2PO_4 concentration). Table 1 summarizes the results of experiments conducted on the basis of the Box–Behnken design. The second-order polynomial equation provides a mathematical model for describing the relationship between the variables and the response. The regression Equation 2 was as follows:

$$Y = 1.18 + 0.14A - 0.023B + 0.13C + 0.024D - 0.0026AB + 0.013AC - 0.02AD - 0.077BC + 0.0069BD + 0.043CD - 0.6A^2 - 0.13B^2 - 0.14C^2 - 0.089D^2 \quad (2)$$

where A is the pH, B is the culture temperature (°C), C is the culture time (h), and D is the KH_2PO_4 concentration (g l^{-1}).

The regression model was statistically significant (P < 0.0001). The lack-of-fit test result (P = 0.6293 > 0.05; not significant) indicated that the mathematical model fits well with the experimental data. The coefficient of determination (R^2) for the model was 0.9589, indicating the model could explain 95.89% of the variability in the data. The adjusted coefficient of determination (R^2_{Adj}) was 0.9177, indicating that the model was a good fit for the data and the errors were small. The influence of each factor on the response value in descending importance was as follows: pH (A) > culture time (C) > KH_2PO_4 concentration (D) > culture temperature (B). The pH and culture time significantly affected the *C. militaris* dry weight, but the culture temperature (P = 0.3966 > 0.05) and

Table 1. Results of the four-factor Box–Behnken design-based experiments.

Source	Sum of squares	df	Mean square	F	P	Significance
Model	2.82	14	0.20	23.31	< 0.0001	**
A	0.23	1	0.23	27.01	0.0001	**
B	6.608E-003	1	6.608E-003	0.76	0.3966	
C	0.2	1	0.20	23.11	0.0003	**
D	7.023E-003	1	7.023E-003	0.81	0.3825	
AB	2.704E-005	1	2.704E-005	3.130E-003	0.9562	
AC	6.812E-004	1	6.812E-004	0.079	0.7830	
AD	1.636E-003	1	1.636E-003	0.19	0.6701	
BC	0.024	1	0.024	2.74	0.1202	
BD	1.904E-004	1	1.904E-004	0.022	0.8841	
CD	7.500E-003	1	7.500E-003	0.87	0.3673	
A^2	2.33	1	2.33	269.40	< 0.0001	**
B^2	0.12	1	0.12	13.32	0.0026	**
C^2	0.12	1	0.12	13.89	0.0023	**
D^2	0.051	1	0.051	5.90	0.0292	*
Residual	0.12	14	8.640E-003	-	-	
Lack of fit	0.082	10	8.182E-003	0.84	0.6293	
Pure error	0.039	4	9.785E-003	-	-	
Cor total	2.94	28	-	-	-	
R^2	0.9589	-	-	-	-	
R^2_{Adj}	0.9177	-	-	-	-	

Note: *significant difference (0.01 < P < 0.05); **extremely significant difference (P < 0.01).

KH_2PO_4 concentration (P = 0.3825 > 0.05) did not. Accordingly, the culture time and pH were the main factors influencing *C. militaris* growth in the liquid culture.

Response surface plots for the interactions among the four factors are presented in Figure 2. The steepness of the response surface curve reflected the significance of the influence. According to these plots, the mycelial dry weight peaked in the tested range. According to the regression model, the optimal liquid culture conditions for *C. militaris* were predicted as follows: pH, 7.56; culture temperature, 20.47 °C; culture time, 110.62 h; and KH_2PO_4 concentration, 1.11 g l^{-1}. Under these conditions, the theoretical mycelial dry weight can reach 1.1199 g l^{-1}. The liquid culture conditions were adjusted to the following: pH, 7.56 ± 0.02; culture temperature, 20.5 ± 0.1 °C; culture time, 110.5 ± 0.5 h; and KH_2PO_4 concentration, 1.11 ± 0.01 g l^{-1}. The experiment was repeated three times, with three replicates per condition, which resulted in an average mycelial dry weight of 1.0226 g l^{-1}. The actual value was close to the predicted value. A previous study showed that mycelial growth, which changes as the culture conditions change, is closely related to the production of active substances (Wen et al., 2014). Therefore, *C. militaris* cultivation conditions should be optimized as much as possible. This study was the first to optimize the culture conditions of *C. militaris* before its injection into *A. pernyi* pupae. Most of the previous studies on the optimization of *C. militaris* culture conditions involved liquid fermentation systems used to produce active substances (Cui & Yuan, 2011; Xie et al., 2009) or long-term *C. militaris* submerged cultures (more than 10 days) used to generate high mycelial yields and promote the production of fermentation by-products. Although *C. militaris* submerged cultures can increase the mycelial weight, long-term hypoxic cultivation will alter gene expression (Suparmin et al., 2017). Moreover, submerged cultures require the continuous addition of nutrients, which increases the possibility of contamination, making them inappropriate for culturing *C. militaris* that will be injected into *A. pernyi* pupae.

3.2 Changes in the fruiting bodies of Cordyceps militaris stromata on Antheraea pernyi pupae after the RSM optimization

The growth of *C. militaris* fruiting bodies on *A. pernyi* pupae after the RSM optimization was analysed. Specifically, the morphological characteristics of the *C. militaris* fruiting bodies on *A. pernyi* pupae before and after the optimization were compared (Figure 3). Before the optimization, there were 17 fruiting bodies (on average), with an average length of 6.9 cm. Some fruiting bodies were light yellow in colour. The length and thickness of the fruiting bodies were uneven, and some fruiting bodies were distorted. After the optimization, there were 22 fruiting bodies (on average), with an average length of 7.9 cm (Figure 4).

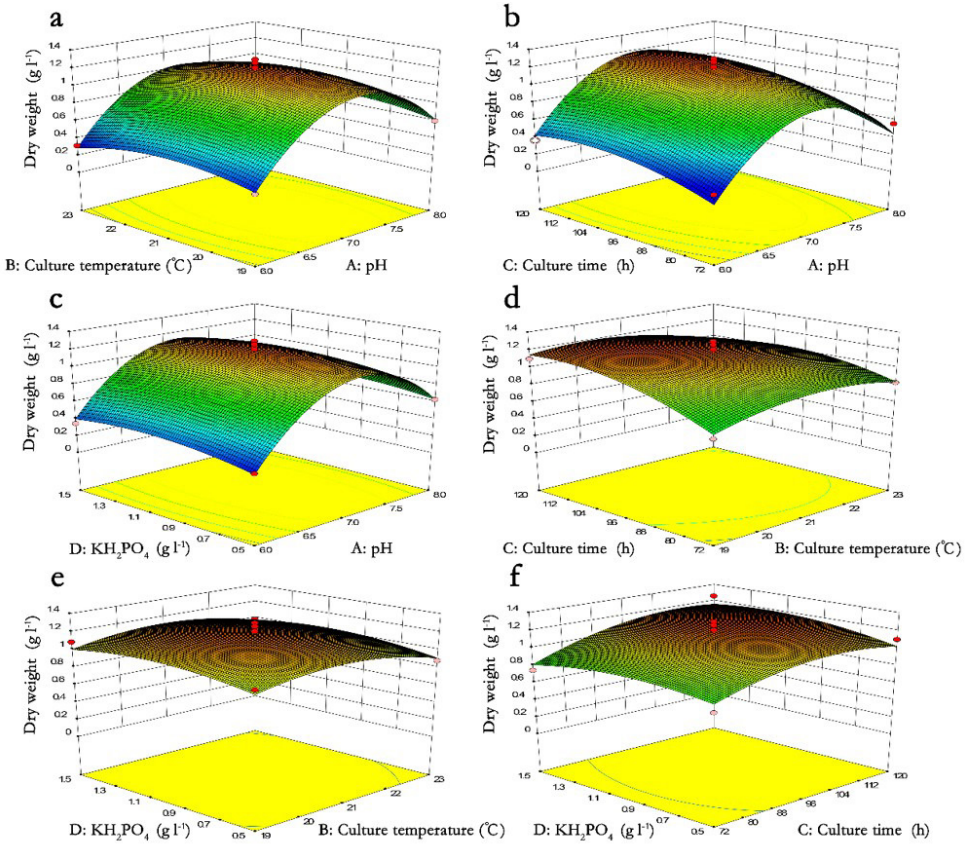

Figure 2. Three-dimensional response surface plots of the interactions among various factors. (a) The interaction of pH and culture temperature. (b) The interaction of pH and culture time. (c) The interaction of pH and KH_2PO_4. (d) The interaction of culture temperature and culture time. (e) The interaction of culture temperature and KH_2PO_4. (f) The interaction of culture time and KH_2PO_4.

Figure 3. Appearance of *C. militaris* fruiting bodies on *A. pernyi* pupae before and after the RSM optimization. (a) Before optimization; (b) After optimization.

The fruiting bodies were dark yellow. The length and thickness of the fruiting bodies were uniform. Hence, the RSM optimization significantly increased the number and length of fruiting bodies ($P < 0.05$). In an earlier study, the optimized *C. militaris* fruiting body length was 5.57 cm (Tao et al., 2021). The significantly greater optimized fruiting body lengths in the current study imply that the RSM optimization of *C. militaris* culture conditions can promote fruiting body growth and colouration. A recent study

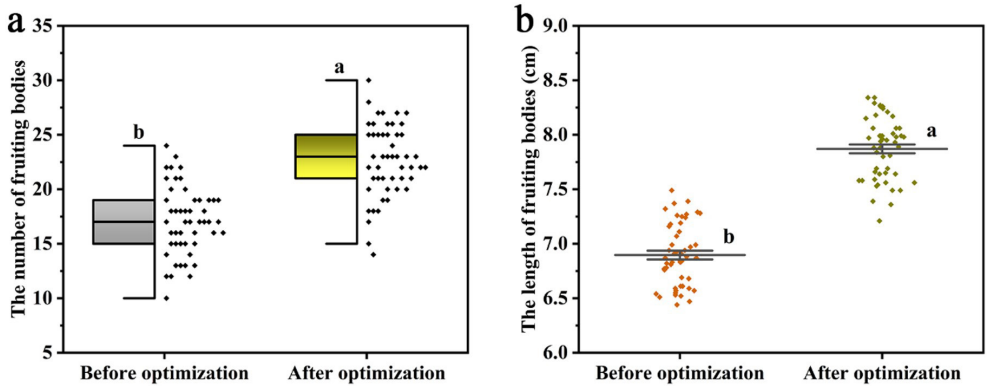

Figure 4. Length and number of *C. militaris* fruiting bodies on *A. pernyi* pupae before and after the RSM optimization. a,b Indicate significant differences (P < 0.05).

Table 2. Active substance contents before and after the RSM optimization.

Active substances	Before optimiation (g kg^{-1})	After optimiation (g kg^{-1})	The increased amount (%)
Adenosine	0.1158 ± 0.0021^{b}	0.1303 ± 0.0009^{a}	12.52
Cordycepin	1.2325 ± 0.0044^{b}	1.3270 ± 0.0043^{a}	7.67
Polysaccharides	5.5457 ± 0.0487^{b}	5.7138 ± 0.0580^{a}	3.03
Carotenoid	2.0093 ± 0.0212^{b}	2.3093 ± 0.0314^{a}	14.93
Cordycepic acid	12.0772 ± 0.1412	12.0797 ± 0.1339	0.02

Note: Different letters in each row indicate significant differences (P < 0.05).

revealed that optimized culture conditions substantially increase *C. militaris* metabolic activities (Yang et al., 2020), which may help to explain the observed increase in the number and length of fruiting bodies (Ha et al., 2022). The production of *C. militaris* stromata on *A. pernyi* pupae is generally assessed in terms of the development and growth of fruiting bodies (Raethong et al., 2020). Ideal culture conditions can promote the synthesis of *C. militaris* fruiting bodies (Nguyen et al., 2019).

3.3 Changes in the active substance contents in Cordyceps militaris stromata on Antheraea pernyi pupae after the RSM optimization

The active substance contents in *C. militaris* stromata on *A. pernyi* pupae before and after the RSM optimization are presented in Table 2. After the optimization, the adenosine content in the *C. militaris* stromata on *A. pernyi* pupae was 0.1303 g kg^{-1}, which was 12.52% higher than the adenosine content before the optimization; this increase was extremely significant (P < 0.01). The cordycepin content after the optimization was 1.3270 g kg^{-1}, which was 7.67% higher than the cordycepin content before the optimization; this increase was extremely significant (P < 0.01). Suitable culture conditions reportedly enhance the accumulation of cordycepin in *C. militaris*. The cordycepin content in *C. militaris* varies depending on the culture temperature and pH (Jiapeng et al., 2014). These findings provide further evidence that optimizing *C. militaris* culture conditions can promote cordycepin production.

The polysaccharide content after the optimization was 5.7138 g kg^{-1}, which was 3.03% higher than the polysaccharide content before the optimization; this increase was significant (0.01 < P < 0.05). Polysaccharide production in *C. militaris* is closely related to metabolic activities, which may be induced by optimizing the culture medium (Zhang et al., 2021).

The carotenoid content after the optimization was 2.3093 g kg^{-1}, which was 14.93% higher than the carotenoid content before the optimization; this increase was extremely significant (P < 0.01). A recent investigation demonstrated that a favourable environment promotes the accumulation of carotenoids in *C. militaris* (Zhao et al., 2021).

The cordycepic acid content was almost unchanged after the optimization, indicating that the RSM optimization did not enhance cordycepic acid biosynthesis. In *C. militaris*, cordycepic acid production is related to metabolic activities, but it is also influenced by the nitrogen and carbon contents in the growth medium (Lin et al., 2016; Lin et al., 2016). In the present study, the nitrogen and carbon contents of the medium were not optimized, which may explain the lack of change in cordycepic acid production.

3.4 Changes in the amino acid content in Cordyceps militaris stromata on Antheraea pernyi pupae after the RSM optimization

The amino acid types and contents in *C. militaris* stromata on *A. pernyi* pupae before and after the RSM optimization were analysed (Figure 5). The number of different types of amino acids (i.e., 18) was unaffected by the RSM optimization. Similarly, there was no significant difference in the total amino

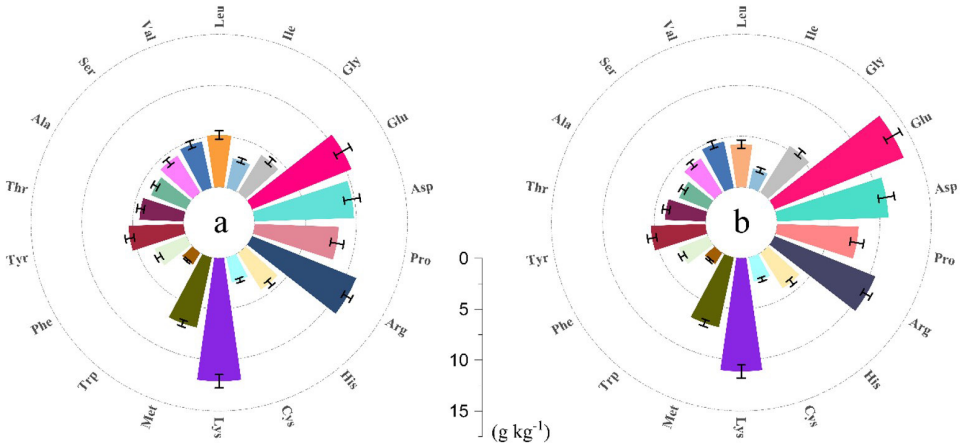

Figure 5. Amino acid contents in *C. militaris* stromata on *A. pernyi* pupae before and after the RSM optimization. (a) Before optimization; (b) After optimization.

acid content in *C. militaris* stromata on *A. pernyi* pupae before (104.98 g kg^{-1}) and after (104.41 g kg^{-1}) the optimization, but the abundance of individual amino acids tended to change to varying degrees. The RSM optimization may have affected amino acid metabolism in *C. militaris*, which is in accordance with the findings of a recent study that confirmed that changes to culture conditions alter the ability of *C. militaris* to metabolize proteins (Huang et al., 2022), while also modulating the abundance of different amino acids. This may help to explain the observed changes to the contents of individual amino acids.

The main reason for the decrease of amino acids is protein synthesis as raw material (Suzuki, 2019). Beyond that, amino acids can be decomposed into ATP by deamination and decarboxylation to provide energy source for metabolic functions (Schinn et al.,, 2021). Amino acids can also provide a carbon skeleton for amino acid synthesis through amino group transformation, from one amino acid to another amino acid, such as glutamic acid converted to aspartic acid under the action of aspartate aminotransferase (Zhou et al., 2022). Different microorganisms have different abilities of deamination, decarboxylation and amino group transformation, which is closely related to the genetic information and culture conditions of microorganisms. *Cordyceps militaris* stromata on *A. pernyi* pupae contain eight essential amino acids that are required by humans. The proportion of essential amino acids was 39% before the optimization and 36% after the optimization (Figure S1). Although the proportion decreased, the total essential amino acid content remained high (37.28 g kg^{-1}). The Food and Agriculture Organization/World Health Organization recommends that adults should consume food containing essential amino acids (Yu et al., 2021). Hence, *C. militaris* stromata on *A. pernyi* pupae may be an excellent source of essential amino acids. Furthermore, the contents of the umami amino acids (UAA) Asp, Glu, and Gly increased significantly after the RSM optimization (Table S2), which was in contrast to the significant decreases in the contents of the sweet amino acids (SAA) Ser, Ala, and Thr (Table S3) and the aromatic amino acids (ArAA) Tyr, Phe, and Trp (Table S4). Although the contents of the bitter amino acids (BAA) Ile and Leu also decreased significantly after the RSM optimization, the Val content was essentially unchanged (Table S5). Significant increases in the UAA content can improve the umami taste of *C. militaris* stromata on *A. pernyi* pupae. Therefore, the optimized *C. militaris* stromata on *A. pernyi* pupae may be useful for developing and producing condiments that are more nutritious than traditional condiments. In addition, Ser, Gly, Ala, and Tyr are important for fat metabolism, muscle growth, and antibody production (Xu et al., 2020). The combined contents of these four amino acids reached 17.69 g kg^{-1} after the optimization. Accordingly, the optimized *C. militaris* stromata on *A. pernyi* pupae may positively affect immune functions.

4 Conclusions

In this study, the liquid culture conditions for *C. militaris* before its injection into *A. pernyi* pupae were optimized on the basis of RSM. The following culture conditions resulted in a mycelial dry weight of 1.0226 g l^{-1}: pH, 7.56 ± 0.02; culture temperature, 20.5 ± 0.1 °C; culture time, 110.5 ± 0.5 h; and KH_2PO_4 concentration, 1.11 ± 0.01 g l^{-1}. The experimental values were similar to the predicted values. After the RSM optimization, the average length of the fruiting bodies increased from 6.9 cm to 7.9 cm. Moreover, the fruiting body colour changed from light yellow to dark yellow. Additionally, the adenosine, cordycepin, polysaccharide, carotenoid, and cordycepic acid contents increased by 12.52%, 7.67%, 3.03%, 14.93%, and 0.02%, respectively, following the optimization. The contents of some amino acids also changed to varying degrees. The study findings will help to increase the quality of *C. militaris* stromata on *A. pernyi* pupae, which will lead to economic benefits for *C. militaris* producers.

Conflict of interest

No conflict of interest was identified.

Availability of data and material

The data underlying this article will be shared on reasonable request to the corresponding author.

Author contributions

J. L. designed and supervised experiments and wrote the manuscript. B. L. and Q. X. conceived the study, analyzed data. K. Q. and J. Z. provided vital reagents and advised about experiments. Z. X. and H. Z. supervised the work and reviewed this manuscript. C. Z., J. H. and Y. J. helped design the study, conducted the initial analyses. All authors have read and agreed to the published version of the manuscript.

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Supplementary Material

Supplementary material accompanies this paper.

Table S1 Factors included in the central experimental design

Table S2 Changes in the UAA content induced by the RSM optimization

Table S3 Changes in the SAA content induced by the RSM optimization

Table S4 Changes in the ArAA content induced by the RSM optimization

Table S5 Changes in the BAA content induced by the RSM optimization

Figure S1 Essential amino acid ratios in *C. militaris* stromata on *A. pernyi* pupae before and after the RSM optimization.

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