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Optimization of *Monochamus alternatus* media and culture period for cordycepin production in *Cordyceps militaris* culture using solid-state fermentation

Si Young Ha, Ji Young Jung, and Jae-Kyung Yang*

Division of Environmental Forest Science, Institute of Agriculture and Life Science, Gyeongsang National University, Jinju, 52828, Republic of Korea

ABSTRACT: In this study, we investigated the effect of solid culture medium on the production of cordycepin in *Cordyceps militaris*. The regression equation was expressed as follows: $Y_1 = 755.3\text{-}58.6625X_1 + 4.79432E\text{-}14X_2\text{-}46.6625X_3\text{-}5.66269E\text{-}14X_1X_2\text{-}0.025X_1X_3 + 1.62475E\text{-}14X_2X_3\text{-}160.6625X_1^2 + 0.0125X_2^2\text{-}206.9625X_3^2$, where, Y represents the value of cordycepin content (μg/g), X_1 corresponds to the weight of *M. alternatus* in solid culture medium (g/bottle), X_2 to the water content of the solid culture medium (%), and X_3 to the culture period (day). The solid culture medium was optimized using the response surface methodology, and the optimal medium composition was as follows: the weight of *M. alternatus* in solid culture medium and water content were 16.2% and 100.7% (20.14 mL water/20 g solid culture medium), respectively, with a culture period of 39 days. Under these conditions, the cordycepin content of the fruiting bodies reached 150.0 μg/g (actual value). The supplementation of *M. alternatus* in solid culture for improved cordycepin content of *C. militaris* seems to be a promising alternative to wild and solid cultivation.

KEYWORDS: Cordycepin, *Cordyceps militaris*, *Monochamus alternatus*, Response surface methodology, Solid-state fermentation

INTRODUCTION

Currently, *Cordyceps militaris* is used as a functional food and medicine in Southeast Asia (López Rodríguez and Burrola-Aguilar, 2019) with the potential to become a medicinal fungus with largest production and popularity in the future. *C. militaris* is at present used as a substitute for *C. sinensis* in traditional medicine as well as in health foods, as the latter is very expensive (Liu *et al*., 2021). Compared to *C. sinensis*, *C. militaris* possesses similar bioactive components; however, it produces a greater quantity of cordycepin (Zhang *et al*., 2020). Furthermore, its significant pharmacological activity has resulted in an increased market demand for *C. militaris* (Li *et al*., 2021).

C. militaris is known for the production of an impressive range of bioactive compounds including polysaccharides, cordycepin, and ergosterol, with significant pharmacological effects (Wu *et al*., 2019). In recent studies, hemagglutinin (Quan *et al*., 2020) and a cytotoxic antifungal protease have been shown to be purified from the dry fruiting body of *C. militaris* (Yu *et al*., 2021).

Cordycepin is a nucleoside derivative isolated from the spent culture media of *C. militaris*, that has drawn considerable interest due to its potent antitumor (Khuntawee *et al*., 2021), and hypolipidemic (Zhang *et al*., 2021) properties. Recently, research has shown that many of the reported bioactive effects of cordycepin are likely to be due to its effects on mammalian target of rapamycin (mTOR) and AMP-activated kinase (AMPK) signaling (Hawley *et al*., 2020).

Techniques for the synthesis of cordycepin involve chemical and biological pathways. Since cordycepin obtained by chemical pathways is difficult to purify with

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Si Young Ha(Ph.D), Ji Young Jung(Research professor), and Jae-Kyung Yang(Professor)
*Corresponding author
E-mail : jkyang68@gmail.com
Tel : +82-55-772-1862,Fax : +82-55-772-1869

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the cost of production being much higher than the biological pathways, the biological pathways have become a major research concern (Zhao *et al*., 2019). There have been many studies that have highlighted the significance of the culture requirements for secondary metabolite production by filamentous fungi (Wösten, 2019).

Similarly, *in vitro* mycelium growth and fruiting body formation in *C. militaris* have attracted the interest of mycologists, entomologists, and biotechnologists. There have been studies on optimizing the culture conditions (Raethong *et al*., 2020) and medium composition (Xu *et al*., 2019) to increase the yield of cordycepin in liquid culture. However, there are only a few reports on the solid-state fermentation of *C. militaris* using insects, except for silkworm pupae.

Monochamus alternatus (Coleoptera: Cerambycidae; *M. alternatus*) popularly known as the Japanese Pine Sawyer, is a vector of pinewood nematode (*Bursaphelenchus xylophilus*) that causes pine wilt disease. This disease is also known as the cancer of pine trees and is responsible for significant environmental and economic losses worldwide (Kim *et al*., 2020). Therefore, controlling *M. alternatus* will help to prevent the spread of pinewood nematodes (Su *et al*., 2020). Moreover, in Korea, *M. alternatus* is currently caught using traps. Rendering the resources of captured *M. alternatus* has numerous advantages, including environmental friendliness, high specificity, and little influence on off-target organisms. So far, there have been no investigations on using *M. alternatus* as growth media on fruiting body formation and cordycepin production in solid-state fermentation by *C. militaris*.

Accordingly, the objective of this study was to optimize *M. alternatus* media and culture period on solid-state fermentation of the growth of *C. militaris* to increase cordycepin via a statistically based experimental design. Medium optimization (*M. alternatus* weight in media, water content of media, and culture period) was performed by a one-factor-at-a-time method, which involved changing one independent variable at a time. Hence, as a more practical method, the orthogonal matrix method was employed to study the relationships between the medium components and their effects on cordycepin production.

MATERIALS AND METHODS

Fungal strains

The *C. militaris* strain (KCCM 60304) was purchased from the Korean Culture Center of Microorganisms (Seoul, South Korea). Fungi were maintained in potato dextrose agar (PDA) medium. *C. militaris* was cultured in PDA medium for 20 days and then subcultured to expand its population for further experiments.

Inoculum preparation

Potato dextrose broth (PDB) medium (50 mL) was poured into 250 mL flasks and autoclaved using pressurized steam at 121°C for 15 min. Each flask was inoculated with the three core mycelial discs (5 mm) of *C. militaris* from growing mycelia on PDA medium. The inoculated media were cultured under static conditions at 25°C for 10 days. The cultured media were then homogenized at 150,000 rpm for 5 min with a homogenizer (AM-11; Nihonseiki Kaisha Ltd., Tokyo, Japan) and filtered through sterilized gauze to remove entangled hyphae.

Solid-state fermentation for growth of fruiting body

In this study, the *M. alternatus* imagos obtained from the Gyeongsangnam-do Forest Environment Research Institute, Jinju, 52615, Republic of Korea were inoculated with *C. militaris* mycelia. *M. alternatus*, with a body length of 20-28 mm, was used as a component of the solid culture medium (Fig. 1). To investigate the effect of *M. alternatus* in solid medium on fruiting body formation, oat solid medium and adults (original or powdered) were mixed with dry weight in the ratios of 7:3 (w:w), 5:5 (w:w), 3 and 7 (w:w). The powder-type *M. alternatus* was obtained after grinding for 60 s using a grinder, and the uniform powder size of 150 μm diameter was prepared using a mesh screen. The solid culture medium of *C. militaris* was prepared in a 300 mL cylindrical plastic bottle (8 cm in diameter and 12 cm in height), sealed with a plastic cap, and autoclaved for 30 min at 121°C. At this time, the water content of the solid culture medium was adjusted to 50%. The solid culture medium with 50%, 75%, and 100% moisture content was prepared by adding 50, 75, and 100 g of distilled water to 20 g of solid culture medium, respectively. The medium was cooled to room temperature, inoculated with 5 mL seed culture, incubated at 24°C for 21 days, and incubated in the dark to promote vegetative growth. The solid culture medium full of hyphae was kept at 20°C under 12 h light and 12 h dark, with light intensity at 1,000 lx for primordial fruiting bodies. The growth chamber for fruit body formation was controlled at a relative humidity of over 60%. The

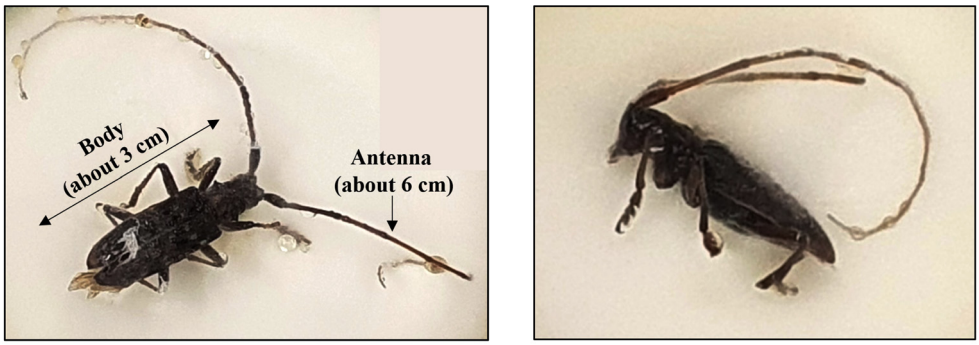

Fig 1. *M. alternatus* used substrate of solid culture medium for *C. militaris* formation.

cultivation conditions were maintained until the top of the fruiting bodies became round and covered with spores. The fruiting body formation was considered to be complete when it no longer grows and there is a head at the end of the fruiting body.

Analytical methods

Fresh weight of the fruiting bodies was determined immediately after harvesting. Fruit bodies obtained from different substrates were dried to a constant weight at 60°C. Dried samples were pulverized to a particle size of less than 20 mesh. For cordycepin extraction, dry powder of 0.5 g (accurate up to 0.0001 g) was suspended in 50 mL of double deionized water and sonicated for 3 h in an ultrasonic bath at 50 kHz, 400 W. The supernatant was obtained by centrifugation at 1,740 x g for 15 min and filtered through a 0.45 μm membrane filter. High-performance liquid chromatography (HPLC) analysis was carried out using an HPLC system (YL9100 plus, YOUNG IN Chromass, Gyeonggi-do, South Korea). An HPLC system with a vacuum degasser, quaternary pump, UV/Vis detector, and analytical software was used for the detection and analysis of cordycepin. The HPLC conditions were as follows: column, Agilent Eclipse plus C18 (250 mm × 4.6 mm, 5 μm); mobile phase, methanol: water (20:80, v/v); flow rate, 1.0 mL/min; UV detection at 260 nm; and injection volume, 10 μL. The samples were filtered through a 0.45 m membrane filter before injection. Cordycepin was quantitatively analyzed using the peak area based on their standard curves. The peaks for cordycepin in the samples were identified by their retention times. At least five replicates were performed for each treatment group. The Box–Behnken design (BBD) of response surface methodology (RSM) was used for optimization of *M. alternatus* weight in solid culture medium, X_1; water content of solid culture medium, X_2; culture period, X_3 and study their interactions. Each effective variable in the design was studied at three different levels (coded as -1, 0, and +1). A total of 17 experiments were conducted, and the entire experimental design considered three center points. The experimental results were fitted with a multiple regression analysis, as explained by a quadratic polynomial equation (Eq. (1)):

$$' = \beta_0 + \Sigma_{i=1}^{4}\beta_i X_I + \Sigma_{i=1}^{4}\beta_{ii} X_i^2 + \Sigma_{i=1}^{3}\Sigma_{i<j}^{4}\beta_{ij} X_I X \quad (1)$$

where, Y is the predicted response (cordycepin content of *C. militaris* fruiting body, μg/g), β0, βi, βii, and βij are constant coefficients, and x_i and x_j are the coded independent variables or factors. The fitness of the second-order polynomial model was expressed via the regression coefficient R^2, and a detailed analysis of variance (ANOVA) was conducted at a coded level of variables to determine their individual effects. SAS software (version 11.0, SAS Institute Inc., Cary, NC, USA) was used for regression and graphical analysis of the experimental data.

Statistical analysis

Data are presented as mean ± standard deviation (n= 3). Statistical analyses of the results were performed at a 5% significance level using the Statistical Analysis System software (SAS Institute, Inc., 2000). Differences between the means of individual groups were assessed using SAS with Duncan's multiple-range test.

RESULTS AND DISCUSSION

Influence of *M. alternatus* form on cordycepin content of fruiting bodies

In this study, the solid culture medium for improved cordycepin content of *C. militaris* fruiting bodies in

Table 1. Test groups according to the form of *M. alternatus* supplement for improved cordycepin content of fruiting bodies

Media[1)]	Oats, g/bottle	*M. alternatus* original form, g/ bottle	*M. alternatus* powdered form, g/ bottle	Total, g/bottle
C1	20	0	0	20
T1	10	10	0	20
T2	10	0	10	20

[1)] C1: only Oats; T1: Oats + *M. alternatus* original form (1:1, w:w based on dry weight); T2: Oats + *M. alternatus* powdered form (1:1, w:w based on dry weight)

solid-state fermentation, including the original and powdered form of *M. alternatus*, was tested (Table 1). Our results showed that the mycelia entirely colonized 300 mL bottles containing 20 g of solid culture medium within 14 days of inoculation.

The oat, including the original form *of M. alternatus*, was found to be the best solid culture medium for improved cordycepin content in the *C. militaris* fruiting body (Table 2). Among the three kinds of solid culture medium, oats with the original form *M. alternatus* gave the highest cordycepin content (82.13 μg/g), while it was 30.91 μg/g when oats including the powder form *M. alternatus* was used. The oats sole had the lowest cordycepin content (0.63 μg/g. In addition, the oats sole or oats including powder from *M. alternatus* was not found to be a good solid culture medium for improved cordycepin content in the *C. militaris* fruiting body. In particular, oats, including the original form *M. alternatus*, were more effective in improving the fruiting body production-based fresh fruit body weight than other solid culture media. Wen *et al*. (2014) have reported that C. *militaris* may find it difficult to utilize grain. Furthermore, in this study, the lowest cordycepin was found to be produced in the solid culture medium using oats alone, which is consistent with previous studies.

Table 2. Effect of *M. alternatus* supplemental form on the cordycepin content of fruiting bodies during solid state fermentation

Media[1)]	Fresh fruiting bodies, g/ bottle	Cordycepin content, μg/ g
C1	19.6 ± 1.1^{b}	0.63 ± 0.00^{c}
T1	52.8 ± 1.5^{a}	82.13 ± 0.05^{a}
T2	14.4 ± 0.1^{c}	30.91 ± 0.01^{b}

[1)] C1: only Oats; T1: Oats + *M. alternatus* original form (1:1, w:w based on dry weight); T2: Oats + *M. alternatus* powdered form (1:1, w:w based on dry weight)
Each value is expressed as mean ± SE (n = 5). Different superscripted letters represent statistically significant probability level at 5%.

Table 3. Test groups according to the weight of *M. alternatus* in media for improved cordycepin content of fruiting bodies

Media[1)]	Oats, g/ bottle	*M. alternatus* original form, g/ bottle	Total, g/ bottle
C1	20	0	20
T1-1	15	5	20
T1-2	10	10	20
T1-3	5	15	20

[1)] C1: only Oats; T1-1: Oats + *M. alternatus* original form (3:1, w:w based on dry weight); T1-2: Oats + *M. alternatus* powdered form (1:1, w:w based on dry weight); T1-3: Oats + *M. alternatus* powdered form (1:3, w:w based on dry weight)

Effect of *M. alternatus* supplements weight on cordycepin content of fruiting body

In this study, the solid culture medium for improved cordycepin content of fruiting bodies in solid-state fermentation, including various supplements of *M. alternatus*, was tested (Table 3). The cordycepin content of *C. militaris* fruiting body was found to be highest in cultures of *C. militaris* supplemented with oats and 15 g/ bottle *M. alternatus* (Table 4). Conversely, the fruiting body yields (based on fresh weight) and cordycepin content were significantly lower ($P = 0.05$) in oats only cultures. It is noteworthy that the fresh weight of the fruiting body was found to be decreased as the feeding weight of *M. alternatus* increased in the growth medium. The fact that the weight of fruiting bodies and cordycepin are inversely proportional has been reported in previous studies as well (Cohen and Nachshol, 2014).

Table 4. Effect of *M. alternatus* weight in media on the cordycepin content of fruiting bodies during solid state fermentation

Media[1)]	Fresh fruit bodies, g/ bottle	Cordycepin content, μg/ g
C1	19.6 ± 1.4^{d}	0.63 ± 0.00^{d}
T1-1	55.0 ± 2.1^{a}	26.28 ± 0.01^{c}
T1-2	52.8 ± 1.6^{b}	82.13 ± 0.05^{b}
T1-3	35.0 ± 1.3^{c}	135.55 ± 0.01^{a}

[1)] C1: only Oats; T1-1: Oats + *M. alternatus* original form (3:1, w:w based on dry weight); T1-2: Oats + *M. alternatus* powdered form (1:1, w:w based on dry weight); T1-3: Oats + *M. alternatus* powdered form (1:3, w:w based on dry weight)
Each value is expressed as mean ± SE (n = 5). Different superscripted letters represent statistically significant probability level at 5%.

Table 5. Test groups according to the water content of *M. alternatus* supplemented culture media for improved cordycepin content of fruiting bodies

Media	Oats, g/ bottle	*M. alternatus* original form, g/ bottle	Water content of media, %
C1W50	20	0	50
C1W75	20	0	75
C1W100	20	0	100
T1-3W50	5	15	50
T1-3W75	5	15	70
T1-3W100	5	15	100

[1)] C1: only Oats, 50% water content; C2: only Oats, 75% water content; C3: only Oats, 100% water content; T1: Oats + *M. alternatus* original form (1:3, w:w based on dry weight), 50% water content; T2: Oats + *M. alternatus* original form (1:3, w:w based on dry weight), 75% water content; T3: Oats + *M. alternatus* original form (1:3, w:w based on dry weight), 100% water content

Therefore, the weight and cordycepin content of the *C. militaris* fruiting body should be optimized, and hence this study focused on improving the cordycepin content.

Effect of solid culture medium water content on cordycepin content of fruiting body

The gas phase, and thus gas exchange, can be altered by adding defined amounts of water to the solid culture medium, which can affect the content of bioactive compounds in the fruiting bodies in mushrooms. Therefore, in this study, changes in the cordycepin content of *C. militaris* fruiting bodies were assessed in relation to the water content of the solid culture medium (Table 5). Our results revealed that water content is a significant parameter affecting the cordycepin content of the *C. militaris* fruiting body (Table 6). Unfortunately, no fruiting bodies were formed in the solid culture medium with 50% water content. However, when the effects of solid culture medium with 100% water content was compared, the cordycepin content of the fruiting body was found to be increased as the water content in the culture medium increased. Zadrail and Brunnert (1981) have reported that the changes in the substrate caused by the metabolic activities of the fungi were related to the water content of the substrate. In our study, the water content of the solid culture medium also influenced the cordycepin content of *C. militaris*. Accordingly, we performed response surface methodology (RSM) based on water content data to find the optimal culture conditions to increase the cordycepin content of fruiting bodies of *C. militaris* using *M. alternatus*.

Table 6. Effect of water content of *M. alternatus* supplemented media on the cordycepin content of fruiting bodies during solid state fermentation

Media	Fresh fruit bodies, g/bottle	Cordycepin content, μg/g
C1W50	Not primordium	Not primordium
C1W75	Not primordium	Not primordium
C1W100	19.6 ± 1.4^{c}	0.6 ± 0.0^{c}
T1-3W50	Not primordium	Not primordium
T1-3W75	20.4 ± 5.1^{b}	111.9 ± 0.0^{b}
T1-3W100	55.0 ± 2.1^{a}	135.6 ± 0.0^{a}

[1)] C1: only Oats, 50% water content; C2: only Oats, 75% water content; C3: only Oats, 100% water content; T1: Oats + *M. alternatus* original form (1:3, w:w based on dry weight), 50% water content; T2: Oats + *M. alternatus* original form (1:3, w:w based on dry weight), 75% water content; T3: Oats + *M. alternatus* original form (1:3, w:w based on dry weight), 100% water content
Each value is expressed as mean ± SE (n = 5). Different superscripted letters represent statistically significant probability level at 5%.

Table 7. Results of three factor Box–Behnken experimental design

Run	Coded			Cordycepin content, μg/g
	X_1	X_2	X_3	Y_1
8	-1	-1	0	64.1
15	1	-1	0	81.5
11	-1	1	0	64.0
4	1	1	0	95.8
1	-1	0	-1	66.9
7	1	0	-1	100.2
3	-1	0	1	60.7
9	1	0	1	75.3
6	0	-1	-1	71.5
16	0	1	-1	72.6
10	0	-1	1	65.3
12	0	1	1	69.8
2	0	0	0	137.2
5	0	0	0	135.9
13	0	0	0	141.1
14	0	0	0	129.5
17	0	0	0	133.5

Independent variables	Code		
	-1	0	1
X_1: *M. alternatus* weight in media, g	10	15	20
X_2: Water content of media, %	75	100	125
X_3: Culture period, day	30	45	60

Table 8. ANOVA results of the fit model from Box-Behnken design

Source	Sum of Squares	Degrees of Freedom	Mean Squares	*F*-Value	*p*-Value	
Model	145.49	9	16.17	46.20	<0.0001	significant
A - X_1	12.75	1	12.75	36.44	0.0005	
B - X_2	0.41	1	0.41	1.16	0.3177	
C - X_3	3.25	1	3.25	9.29	0.0186	
AB	0.49	1	0.49	1.40	0.2753	
AC	1.82	1	1.82	5.21	0.0565	
BC	0.04	1	0.04	0.11	0.7452	
A^2	27.54	1	27.54	78.70	<0.0001	
B^2	45.37	1	45.37	129.65	<0.0001	
C^2	40.66	1	40.66	116.19	<0.0001	
Residual	2.45	7	0.35			
Lack of Fit	0.86	3	0.29	0.72	0.5909	
Pure Error	1.59	4	0.40			
Corrected Total Sum of Squares	147.94	16				

Optimization of screened variables using RSM (BBD) for improved cordycepin content of fruiting body

RSM analysis was performed to obtain the maximum fruiting body dry weight of *C. militaris* using our previous results. The BBD was used to optimize the levels of significant variables (*M. alternatus* weight in solid culture medium, water content of solid culture medium, and culture period). The actual values of the 17 BBD experiments are listed in Table 7.

Our analyses revealed that a second-order polynomial equation provided a mathematical model to describe the relationship between the variables and the response. The regression equation is expressed as follows:

$$Y_1 = 755.3-58.6625X_1+4.79432E-14X_2-46.6625X_3-5.66269E-14X_1X_2-0.025X_1X_3+1.62475E-14X_2X_3-160.6625X_1^2+0.0125X_2^2-206.9625X_3^2$$

where, Y represents the value of cordycepin content (μg/g), X_1 is the *M. alternatus* weight in solid culture medium (g/bottle), X_2 is the water content of the solid culture medium (%), and X_3 is the culture period (day). The ANOVA of the optimization indicated the response surface model terms, which are enlisted in Table 8. The coefficient of determination (R^2) for the model was 0.9772, which could explain the 97.72% variability in the modeling data. The F-value of the model was 46.20, and the *p* value was < 0.0001, which indicates that the experimental data fit well with the quadratic model. The regression coefficients *F* value, *p* value and standard error were summarized and selected for ANOVAs of the model to analyze the relationship between the cordycepin content and each variable (Table 8), indicating the variables of square interaction (A^2, B^2 and C^2 source) were all significant on the cordycepin content.

Three-dimensional response surface plots and corresponding contour plots were used to optimize the levels of all variables for cordycepin content in the *C. militaris* fruiting body (Fig. 2). From the response surface plots and contour plots, the cordycepin content of the fruiting body was found to be highest in the tested range.

Based on the aforementioned equation and the response surface plots, the optimum levels of the three variables were as follows: the *M. alternatus* weight in solid culture medium and water content were 16.2% and 100.7% (20.14 mL water /20 g solid culture medium), respectively, and the culture period was 39 days. The maximum value of cordycepin content in the fruiting body, which could be obtained by the model, was 148.2127 μg/g. Accordingly, the final optimized medium contained 16.2 g/bottle *M. alternatus* weight in solid culture medium, 3.8 g oats, and 100% water content. In order to validate the predicted results of the statistical model, experiments using the optimized medium composition were conducted, which yielded a cordycepin content of 150.0 μg/g. This result thus, confirms the validity of the

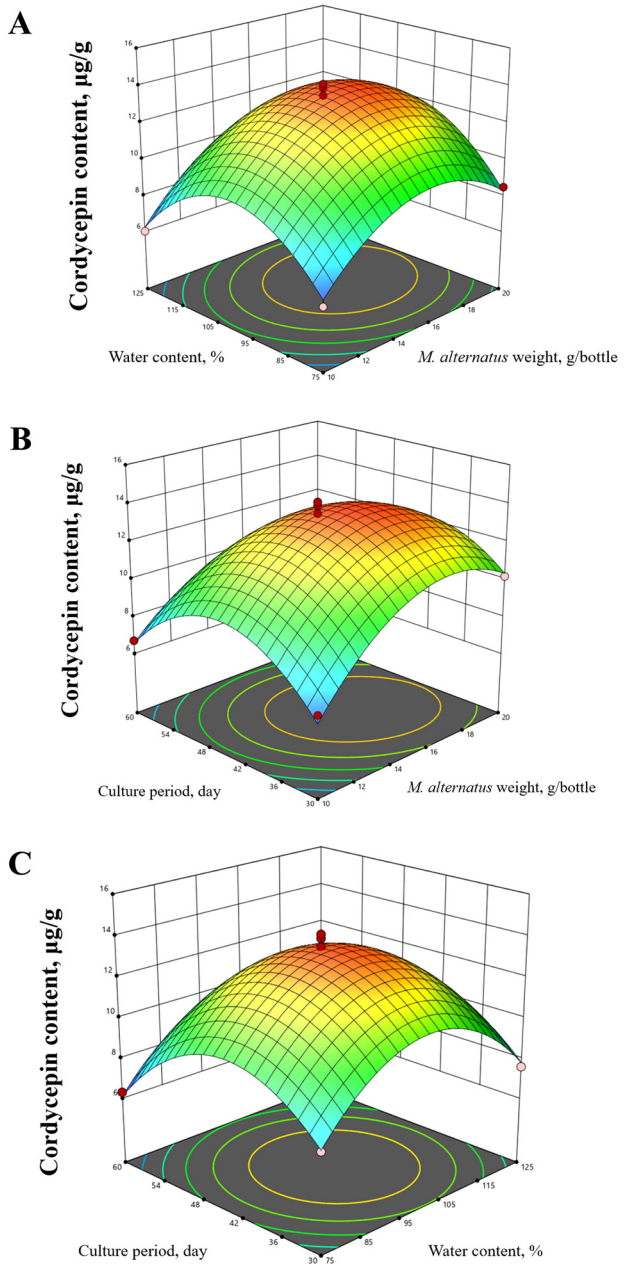

Fig. 2. Regression analysis of the Box–Behnken design experiments. A: response surface graphs for cordycepin content as a function of *M. alternatus* weight in media and water content of media; B: response surface graphs for cordycepin content as a function *M. alternatus* weight in media and culture period; C: response surface graphs for cordycepin content as a function of water content of media and culture period.

model in simulating and predicting the value of cordycepin in the *C. militaris* fruiting body upon using the optimized growth media and conditions. Until recently, relatively low levels of cordycepin content have been produced in solid-state fermentation of *Cordyceps* sp.; 98.0 μg/g cordycepin content was reported in solid-state fermentation (solid culture medium containing 15 g pupae) of *Cordyceps* (Kim *et al*., 2018). The maximum cordycepin content (150.0 μg/g, actual value) of the fruiting body obtained in the present study is significantly higher than those reported previously. In conclusion, supplementation of *M. alternatus* for improved cordycepin content in *Cordyceps militaris* is a promising alternative to wild and solid cultivation.

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