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Research Article

Evaluating the Effects of Different Nutritional Conditions on Fruiting Body Development and Extraction Efficiency of *Cordyceps militaris*

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ABSTRACT

Cordyceps militaris is a valuable medicinal mushroom due to its bioactive components, especially cordycepin and adenosine, which have significant pharmacological activities. However, the yield and quality of fruiting bodies and their active compounds can vary greatly depending on the nutritional conditions during cultivation. This study aimed to determine the optimal culture medium and extraction procedure to enhance both biomass yield and bioactive compound content in *C. militaris.* The mushroom was cultivated under three nutritional conditions: Basal control medium, T1 medium supplemented with soybean powder and coconut water, and T2 medium supplemented with mineral salts ($MgSO_4$, K_2SO_4) without soybean powder or coconut water. Growth performance, fruiting body morphology, and cordycepin/adenosine content were evaluated, followed by optimization of extraction efficiency using Box–Behnken design, taking into account extraction temperature, ethanol/mushroom ratio, and extraction time. T1 provided the most favorable conditions for the development of *C. militaris* fruiting bodies compared to the control group, including the indices stem length (9.82 ± 1.44 cm), diameter (0.83 ± 0.16 cm), number of fruiting bodies (185.88 ± 19.53), and weight (17.18 ± 2.73 g). HPLC analysis showed the highest content of cordycepin (337.93 ± 15.08 mg/100 g) and adenosine (394.51 ± 10.86 mg/100 g). Therefore, we used group T1 to evaluate the effects of temperature, ethanol/fungi ratio, and extraction time on extraction efficiency (17 treatments). The results showed that treatment 17, with the conditions of 60°C, ethanol/fungi ratio 20:1, 6 hr, gave the best extraction efficiency of 33.15%. These results showed that the addition of nutrients from soybean and coconut water and the factors (temperature, ethanol/fungi ratio, and extraction time) significantly affected both the biomass and bioactive compound content of *C. militaris* as well as the extraction efficiency, providing a basis for improving the large-scale cultivation and extraction process for functional food and pharmaceutical applications.

Key words: Adenosine, *Cordyceps militaris*, cordycepin, coconut water, soybean

INTRODUCTION

Cordyceps militaris is a species within the genus *Cordyceps,* comprising entomopathogenic fungi that parasitize the nymphs, pupae, or adults of various insect hosts (Paterson *et al*., 2008). It is widely cultivated on an industrial scale in many Asian countries and is commonly utilized as a functional food, health supplement, cosmetic ingredient, and traditional remedy in China, Japan, Korea, and Southeast Asia (Dong *et al*., 2013; Liu *et al*., 2018). *C. militaris* is recognized for its diverse repertoire of bioactive constituents, including adenosine, cordycepin, cordycepic acid (D-mannitol), nucleic acids, and polyphenols (Sakao *et al*., 2024). It also provides essential vitamins such as B1, B2, and B12; carbohydrates; polysaccharides; proteins; sterols; nucleosides; and trace minerals such as selenium (Se), zinc (Zn), and iron (Fe) (Dong *et al*., 2013; Sakao *et al*., 2024). A lot of research has shown that *C. militaris* exhibits diverse pharmacological activities, including anti-tumor, immunostimulatory, anti-inflammatory, and hypoglycemic effects (Sakao *et al*., 2024). Among its constituents, cordycepin and adenosine are considered the most abundant and nutritionally significant bioactive molecules (Du *et al*., 2021; Sakao *et al*., 2024). Cordycepin, a water-insoluble organic compound, has been reported to possess antioxidant, immunostimulatory, anti-inflammatory, anti-tumor, steroidogenic, and spermatogenic properties (Jędrejko *et al*., 2021). Jędrejko *et al*. (2021) demonstrated its immunomodulatory effects through increased numbers of T (CD4+) and T (CD8+) cells, as well as increased secretion of interleukins and factors associated with both Th1 and Th2 responses (Qin *et al*., 2019; Jędrejko *et al*., 2021). Adenosine is recognized as the principal pharmacologically active component of *C. militaris*. It comprises adenine and D-ribose, which are crucial for numerous cellular processes, including DNA and RNA synthesis. Adenosine also exhibits cardioprotective properties and has been utilized therapeutically in the management of persistent cardiovascular disease. In addition, it contributes to the maintenance of central nervous system equilibrium by modulating neurotransmitter release and neural signaling pathways (Dong *et al*., 2012; Liu *et al*., 2018; Singpoonga *et al*., 2020). Notably, the adenosine A3 receptor, which can bind cordycepin, is overexpressed in many cancer cell types and has shown potential in tumor inhibition (Sakao *et al*., 2024). Due to its rich nutritional and medicinal

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properties, *C. militaris* has garnered significant attention for research and development at both the laboratory and industrial levels. Additionally, artificial cultivation techniques have facilitated quality control, enhanced productivity, and reduced harvesting pressure on natural populations.

Over the past few years, the cultivation of *C. militaris* has witnessed substantial growth globally, driven by increasing demand for its utilization in agriculture, pharmaceutical industries, and functional food production (Liu *et al*., 2018; Sakao *et al*., 2024). Artificial cultivation has become the predominant method for *C. militaris* production, offering an effective and essential solution to address challenges associated with large-scale industrial production and to reduce overexploitation of natural resources (Tang *et al*., 2018; Raethong *et al*., 2020; Kontogiannatos *et al*., 2021). Studies have shown that the concentrations of cordycepin, adenosine, and polysaccharides in fruit bodies can be enhanced through the supplementation of nutrients during solid-state fermentation, liquid fermentation, and host-based cultivation. A variety of research has been undertaken to enhance both the quality and yield of fruit bodies through solid-state fermentation (Lee *et al*., 2016; Lin *et al*., 2017; Raethong *et al*., 2020), liquid fermentation (Cui *et al*., 2015; Tang *et al*., 2018), and insect-based substrate cultivation using silkworm pupae (Guo *et al*., 2016). Carbon, nitrogen sources, and trace elements such as Mg^{2+} and K^{+} are essential components that provide energy and support the growth of microorganisms as well as the biosynthesis of secondary metabolites (Tang *et al*., 2018; Rózsa *et al*., 2021). Soybean powder is a rich source of plant-derived nitrogen, containing approximately 35–40% protein along with carbohydrates, lipids, fiber, and sugars. This composition makes it an excellent nutrient source for microbial growth (Malik *et al*., 2022). Notably, soybean powder extract has been employed as a supplemental nitrogen source and has been reported to stimulate mycelial growth and enhance cordycepin accumulation in *Cordyceps militaris* cultures (Sripilai *et al*., 2023). Coconut water is a carbon-rich substrate, comprising sugars, lipids, amino acids, mineral salts such as calcium, phosphorus, and iron, as well as B vitamins and vitamin C. Consequently, it has been widely applied in plant tissue culture, fungal cultivation, and bacterial growth (Kuntiya *et al*., 2010; Sekar *et al*., 2013). Shashidhar *et al*. (2017) demonstrated that the use of coconut water in submerged cultivation of Ophiocordyceps sinensis improved biomass yield (2.2–2.5-fold), as well as adenosine (58%–69%) and cordycepin (50%–55%) content. More recently, Chellapandi *et al*. (2024) employed coconut water as a culture component in studies on O. sinensis. However, investigations into the use of coconut water in *C. militaris* cultivation, as well as the potential synergistic effects of its combination with soybean powder, remain limited.

Therefore, the present study aims to evaluate the effects of nutritional supplementation with soybean powder and coconut water on the growth performance, fruiting body development, and production of bioactive compounds (cordycepin & adenosine) in C. militaris. Furthermore, this study seeks to optimize extraction efficiency using a Box–Behnken experimental design, focusing on the influence of extraction temperature, ethanol-to-mushroom ratio, and extraction time. Ultimately, the findings will contribute to the selection of the most efficient cultivation protocol, thereby enhancing the value and quality of the product while deepening our understanding of this medicinal fungus.

MATERIALS AND METHODS

Materials

C. militaris was provided by the University of Education, Hue University. The fungus was cultured on potato dextrose agar (PDA) medium at 22°C and 80% humidity to monitor the growth of mycelium. After the mycelium covered the surface of the medium plate, a first generation of culture on a 1 × 1 cm^2 PDA medium was placed in a PDB culture flask, then incubated at 22°C with a shaking speed of 150 r.p.m.

Evaluation of fruiting body development on synthetic medium

After incubation and shaking, the fungal strain was transferred to the fruiting body culture (FBC) medium. Fruiting bodies of *C. militaris* were made using previously reported techniques, with minor adjustments. (Wen *et al*., 2012). The experiment was conducted to evaluate the efficiency of mycelial growth and fruiting body formation of *C. militaris* using three different culture media formulations.

Basal medium (control): 200 g/L potato extract, 30 g brown rice/jar, 200 g/L silkworm pupae, yeast 2 g/L, peptone 2 g/L. Treatment 1 (T1): 200 g/L soybean powder, coconut water 50 mL/L, MgSO4 0.55 g/L, K2SO4 1.1 g/L, Dextrose 22 g/L, 30g brown rice/jar, 200 g/L silkworm pupae, yeast 2 g/L, peptone 2 g/L. Treatment 2 (T2): 200 g/L potato extract, MgSO4 0.55 g/L, K2SO4 1.1 g/L, 30 g brown rice/jar, 200 g/L silkworm pupae, yeast 2 g/L, peptone 2 g/L.

FBC medium was placed in 10x9x9 diameter plastic lidded boxes. The boxes were then kept in the dark to allow the mycelium to completely colonize the surface of the medium. Next, the boxes were exposed to light to induce primordia formation, 80-90% humidity, and 22°C to promote fruiting body development. The fungal growth was monitored at different stages for 60 days. The *C. militaris* fruiting body characteristics monitored included: Stem length (cm), diameter (cm), number of fruiting bodies, and weight (g). All media were autoclaved at 121°C for 2h, and the experiment was repeated three times (Wen *et al*., 2012).

Determination of bioactive compound content by HPLC

Following harvest, 1 g of *C. militaris* was dried, crushed, and extracted using 10 mL of methanol-water (50/50, V/V) with an ultrasonic extraction machine set to 50 KHz for 30 min. Before HPLC analysis, the material was passed through a 0.45 µm filter.

HPLC analysis was carried out using a modified version of Huang *et al*. (2009)'s methodology. A Waters 2695 Separation Module (Waters, Milford, MA, USA) consisting of a Waters 2996 Photodiode Array Detector, an autoinjector, and a C18 reversed phase column (250 × 4.6 mm; 5 µm) was used. Mobile phase, methanol: water (15:85, v/v); flow rate, 0.8 mL/min; Measurement at 260 nm; and injection volume, 10 µL. Standards for adenosine and cordycepin were acquired from Sigma Chemical Corporation (St. Louis, USA).

Evaluation of *C. militaris* culture performance

To improve the *C. militaris* extraction procedure of fruiting bodies. We selected the most effective fruiting body culture process from previous experiments. Using Box-Behnken design (Ferreira *et al*., 2007) to evaluate the influence of extraction temperature (X1, 50- 70°C), ethanol/fungi ratio (X2, 10:1-20:1), and extraction time (X3, 4-6 hr) to determine the ideal extractive process parameters. The factors were coded as 0 (center value), -1, and +1 (marginal value equidistant from the center value). The experiment was repeated 3 times and analyzed using Design Expert software (version 13). Predicted productivity, the response variable, is a polynomial of the 2nd order model, as seen below:

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i^2 + \sum \sum_{i<j} \beta_{ij} X_{ij} X_i X_j$$

Which y is predicted productivity; β_0 is the regression coefficient for main; X_i is independent factor that effects on y; β_i is the regression coefficient level 1 that describes the influence of X_i on y; β_{ii} is the interactive regression coefficient describing the effect of X_i on y; β_{ij} is the interactive regression coefficient describing the simultaneous effect of X_i and X_j on y, k is factors surveying in experimental design (*k*=3). The experimental productivity of the *C. militaris* extraction process is calculated as H (%) = (m_{dr}/mi) × 100 (g/g), where m_{dr} is the mass of dry extract and m_i is the mass of initial fruiting bodies of fungi.

Statistical analysis

The mean ± SD is used to represent the values. Tukey's test was used after analysis of variance (ANOVA) for post hoc comparisons (*P*<0.05). Version 20 of the SPSS software program was used to analyze the statistical data. The tests were designed using the Design Expert software program 13 (Stat-Ease Inc., Minneapolis, USA), and the experimental data were analyzed graphically and by regression. All experiments were conducted in triplicate.

RESULTS

Culture morphology

After liquid culture, *C. militaris* was transferred to control, T1, and T2 media under dark conditions. The fungal development was monitored at different time points (3, 5 & 7 days) to evaluate the impact of the media on mycelial development. The results indicated that, for each nutritional medium, the growth and development of the mycelial network, as well as fruiting body formation, varied. By day 3, hyphal structures began to form and expanded across the surface of the substrates. This development signified spore germination and the initiation of primary hyphal formation.

By day 5, the mycelium had spread across the surface of the culture medium, forming a thin white layer. By day 7, differences in mycelial density among the experimental groups were observed. In the T1 and T2 treatments, the mycelia formed dense, cotton-like clusters, whereas the control group maintained only a thin, uniform layer on the culture surface.

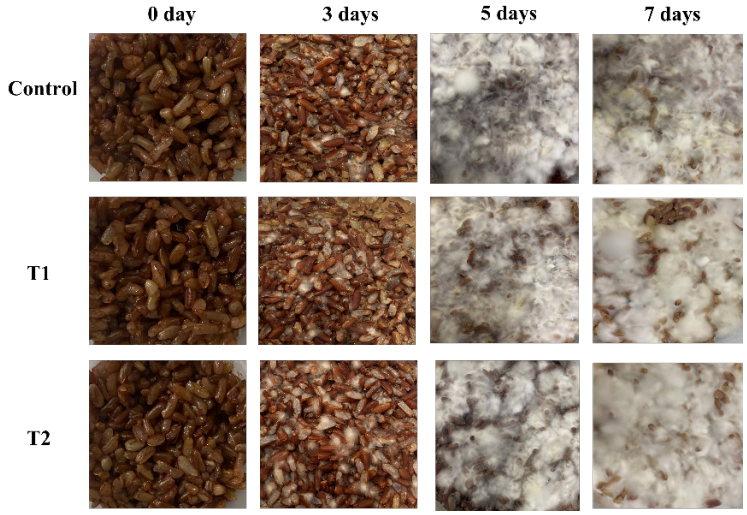

Fig. 1. Growth process of *C. militaris* mycelium in control groups, T1 and T2. Results were monitored for 3 days, 5 days, and 7 days.

Fruiting body development

After the dark incubation phase, the control and treatment groups (T1 & T2) received access to a 12-hr light period each day, with a steady temperature of 22°C and a humidity of 80-90%. These conditions were intended to induce the yellow pigmentation of the mycelium, promote primordia initiation, and support fruiting body development. Morphological development of *C. militaris* was assessed at 4, 7, 15, 30, 45, and 60 days to evaluate the impact of different treatments on hyphal growth and fruiting body formation.

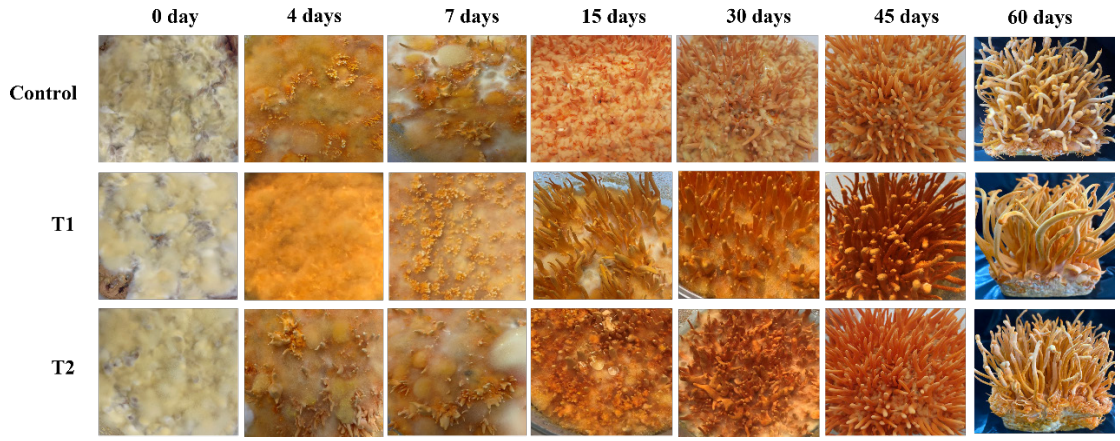

Fig. 2. Mycelial growth and development of *Cordyceps militaris* in different experimental groups (control, T1 & T2) were observed at 4, 7, 15, 30, 45, and 60 days of cultivation.

After 4 days of exposure to light, the color of *C. militaris* mycelia in all groups (control, T1 & T2) had turned to the characteristic yellow. In the T1 group, the mycelial density was high, with uniformly bright yellow pigmentation and the emergence of initial primordia on the surface of the substrate. In contrast, although mycelia in the control and T2 groups also turned yellow, the pigmentation was lighter compared to T1, and some areas still displayed white mycelia. Interestingly, despite the lighter pigmentation, the primordia in these two groups were more prominent than in T1, forming distinct clusters that were easier to observe (Figure 2). By day 7, morphological differences among the groups became more apparent. In the control group, the mycelial pigmentation remained uneven, and the primordia appeared sparsely and irregularly. In the T2 group, primordia formed in larger and more concentrated clusters than in the control. Remarkably, T1 exhibited a significant difference from both the control and T2; the primordia were dense, evenly distributed, and fully covered the surface of the culture medium, with uniform size development (Figure 2). These results suggest that the supplementation of additional substrates, such as nitrogen and carbon sources, contributed to the observed differences between the control and treatment groups. Such nutrient enrichment provided a favorable foundation for the initiation of fruiting body development.

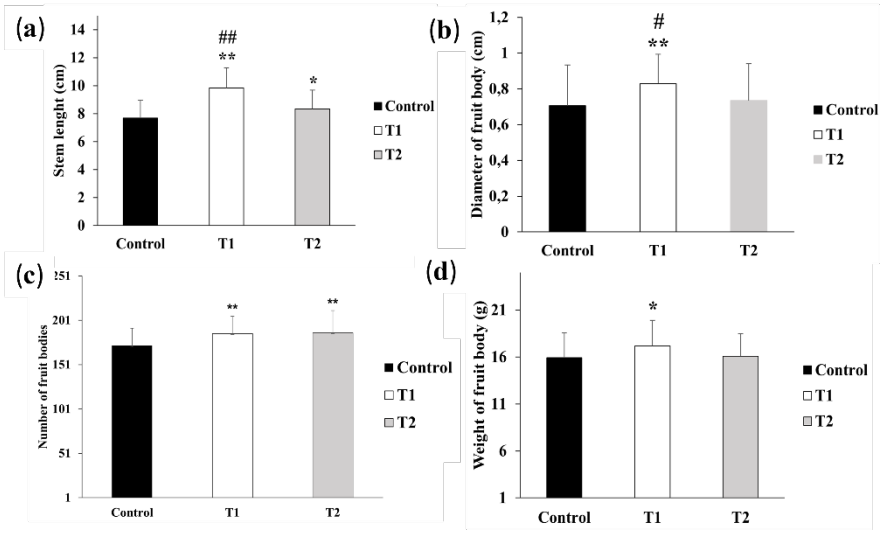

Fig. 3. Morphological indices of *C. militaris* fruiting bodies after 60 days of cultivation. (a) Stem length (cm); (b) Diameter of fruiting body (cm); (c) Number of fruiting bodies; (d) Weight of fruiting body (g). **P*<0.05; ***P*<0.01 represents a statistically significant difference between the treatment group (T1 & T2) and the control group. #*P*<0.05; ##*P*<0.01 represents a statistically significant difference between the T1 and T2 groups. The experiment was repeated three times.

After 60 days of cultivation, the morphological characteristics of *C. militaris* fruiting bodies showed significant differences among the control, T1, and T2 groups. Regarding fruiting body length, both treatment groups exhibited statistically significant increases compared to the control (7.69 ± 1.28 cm), with T1 (9.82 ± 1.44 cm) and T2 (8.34 ± 1.33 cm) being 1.27-fold (***P*<0,01) and 1.08-fold (**P*<0.05) longer, correspondingly. Furthermore, T1 exhibited a stem length that was 1.17-fold greater than T2 (##*P*<0.01) (Figure 3a). Similarly, significant differences were observed in fruiting body diameter. Although the control (0.70 ± 0.22 cm) and T2 (0.73 ± 0.20 cm) did not vary significantly (*P*>0.05), T1 (0.83 ± 0.16 cm) displayed a significantly greater diameter than both groups (***P*<0.01; #*P*<0.05) (Figure 3b). Notably, fruiting bodies of T1 exhibited a more pronounced cap structure compared to the slender shape retained in the control group. In terms of fruiting body number, both T1 (185.88 ± 19.53) and T2 (186.68 ± 25.10) were significantly higher than the control (172.56 ± 19.44) (***P*<0.01), even though there was no discernible change between T1 and T2 (*P*>0.05) (Figure 3c). Regarding fruiting body weight, the difference between T1 (17.18 ± 2.73 g) and the control

(15.91 ± 2.64 g) was statistically significant (*P<0.01), whereas the difference between T2 (16.07 ± 2.40 g) and the control was not significant (P>0.05) (Figure 3d). Even yet, there was no discernible difference among the treatment groups; the T1 group showed a consistent trend toward greater improvement. Therefore, T1 is considered a more effective condition for enhancing the biomass yield of *C. militaris* fruiting bodies. These findings provide credence to the idea that giving *C. militaris* soybean powder supplements encourages the growth of fruiting bodies.

Cordycepin and adenosine content

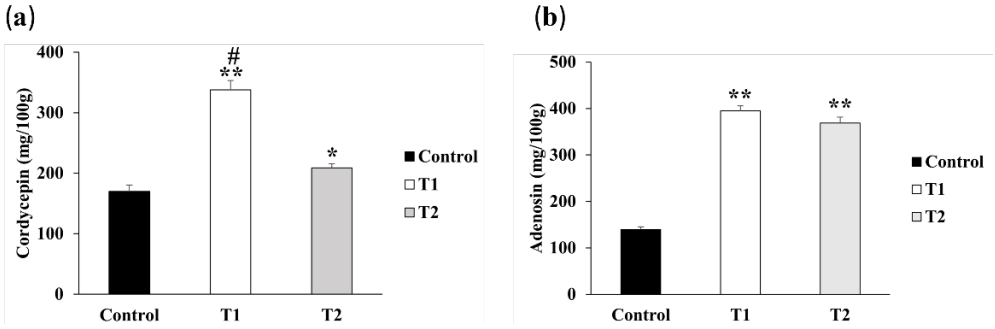

Fig. 4. Cordycepin and adenosine content after 60 days of culture. (a) Cordycepin (mg/100g); (b) Adenosine (mg/100 g). *P<0.05; **P<0.01 represents a statistically significant difference between the treatment group (T1 & T2) and the control group. #P<0.05; ##P<0.01 represents a statistically significant difference between the T1 and T2 groups. The experiment was repeated three times.

After 60 days, we evaluated the cordycepin and adenosine concentrations in the three study groups. The findings demonstrated that the control, T1, and T2 study groups differed statistically significantly. According to the HPLC results, both treatment groups' cordycepin contents were statistically significantly higher than those of the control group (169.80 ± 10.62 mg/100 g), with T1 (337.93 ± 15.08 mg/100 g) and T2 (208.49 ± 6.93 mg/100 g) showing increases of 1.98-fold (**P<0,01) and 1.22-fold (*P<0,05), respectively (Figure 4a). In addition, T1 also showed a statistically significantly higher cordycepin concentration than T2 (#P<0.05). Similarly, the adenosine concentration in both treated groups exhibited a notable and statistically significant rise relative to the control group (139.28 ± 6.13 mg/100 g), with T1 (394.51 ± 10.86 mg/100 g) and T2 (368.80 ± 12.78 mg/100 g) showing increases of 2.83-fold (**P<0.01) and 2.64-fold (**P<0.01), respectively (Figure 4b). The adenosine concentration in T1 and T2 did not differ significantly from each other. Substrate supplementation for the culture process showed differences in cordycepin and adenosine content. The present results showed that T1 was considered a more effective culture condition in enhancing the nutrient content in the fruiting bodies of *C. militaris*.

Culture efficiency of *C. militaris*

The aforementioned findings on fruiting body development in *C. militaris* indicated that treatment T1 was the most promising. Therefore, in the next study, we used treatment T1 to study the extraction efficiency. The influencing factors were evaluated through three levels, as shown in Tables 1 and 2.

Table 1. Coded level of factors used in the study

Factor	Symbol	Range		
Extraction temperature	X_1	50	60	70
Ethanol/fungi ratio	X_2	10	15	20
Extraction time	X_3	4	5	6

For *C. militaris* fruiting bodies, to improve the extraction procedure, the factors of extraction temperature (X_1: 50–70°C), ethanol/fungi ratio (X_2: 10:1–20:1), and extraction time (X_3: 4–6 h) were evaluated using Box-Behnken. ANOVA revealed that the exponential regression system was the finest fit to describe the relationship between independent variables and extraction efficiency (P<0.01). The Model F-value of 13.15 and P-value<0.05 (0.0013) indicate significant model terms. In this context, X1, X2, X3, and X2² were identified as statistically significant terms in the model. P-values>0.05 indicated non-significant model terms (Table 3). Statistical results showed that the ethanol/fungi ratio (X2; F=49.77; P=0.0002) was the element that had the strongest influence on extraction efficiency, afterward by extraction time (X3, F=14.61; P=0.0065), and extraction temperature (X_1, F=9.35; P=0.0184). Similarly, factor X_2^2 (F=31.04; P=0.0008) also showed a statistically significant influence. On the contrary, factors (X_1X_2, X_1X_3, X_2X_3, X_1^2, X_3^2) were not statistically significant (P>0.1). This result showed that the solvent ratio strongly influenced the extraction efficiency, and the impact of each factor was relatively independent.

Table 2. The effect of three factors on extraction efficiency

Treatment	Temperature (^{0}C)	Ethanol/fungi ratio	Extraction time (h)	Efficiency (%)
1	60	10	6	26.4367
2	60	20	4	28.2667
3	50	20	5	28.1967
4	70	10	5	26.2600
5	60	15	5	29.4867
6	50	15	6	29.8800
7	60	10	4	24.8067
8	70	15	6	31.2367
9	50	10	5	25.4933
10	70	20	5	31.4267
11	60	15	5	31.5867
12	50	15	4	27.6933
13	70	15	4	30.1633
14	60	15	5	31.2933
15	60	15	5	31.8400
16	60	15	5	31.6800
17	60	20	6	33.1533

Among the 17 treatments conducted, treatment 17 showed the most optimal efficiency (33.15%) with an extraction temperature of 60°C, ethanol/fungi ratio of 20:1, and extraction time of 6 hr (Table 2). Meanwhile, the remaining 16 treatments had efficiencies ranging from 24.80% to 31.84%.

Table 3. Analysis of variance for the simplified quadratic model

Source	Sum of Squares	df	Mean Square	*F*-value	*p*-value	
Model	96.78	9	10.75	13.15	0.0013	significant
X_1- temperature	7.65	1	7.65	9.35	0.0184*	
X_2- ethanol/fungi ratio	40.71	1	40.71	49.77	0.0002**	
X_3- extraction time	11.95	1	11.95	14.61	0.0065**	
X_1X_2	1.52	1	1.52	1.85	0.2154	
X_1X_3	0.3099	1	0.3099	0.3788	0.5577	
X_2X_3	2.65	1	2.65	3.24	0.1148	
X_1^2	3.24	1	3.24	3.97	0.0867	
X_2^2	25.38	1	25.38	31.04	0.0008*	
X_3^2	1.30	1	1.30	1.59	0.2474	
Residual	5.73	7	0.8179			
Lack of Fit	1.99	3	0.6647	0.7126	0.5935	not significant
Pure Error	3.73	4	0.9328			
Cor Total	102.51	16				

Adj R^2=0.8723; Pre R^2=0.6319; *0.05 significance level; **0.01 significance level

The 3D surface graph and its associated contour diagram were used to illustrate the interaction among extraction temperature (°C), ethanol/fungi ratio, and extraction time (hr) in relation to the extraction efficiency of bioactive compounds from *C. militaris*. The corresponding contour graphic illustrates how temperature and time to extract interact (Figure 5a), in which the X axis (extraction time) and Y axis (extraction temperature) in the model plot it shows that the region with the highest efficiency (above 32%) corresponds to the X value range from 60 to 65°C and the Y range is about 6 hr. Similarly, when the temperature value is about 60 to 65°C and the ethanol/fungi ratio is from 18:1 to 20:1 (Figure 5b), its impact factor on the efficiency is above 30%. When the ethanol/fungi ratio is from 18:1 to 20:1 and the extraction time is from 5.5 hr to 6 hr (Figure 5c), its impact factor on the efficiency is above 33%.

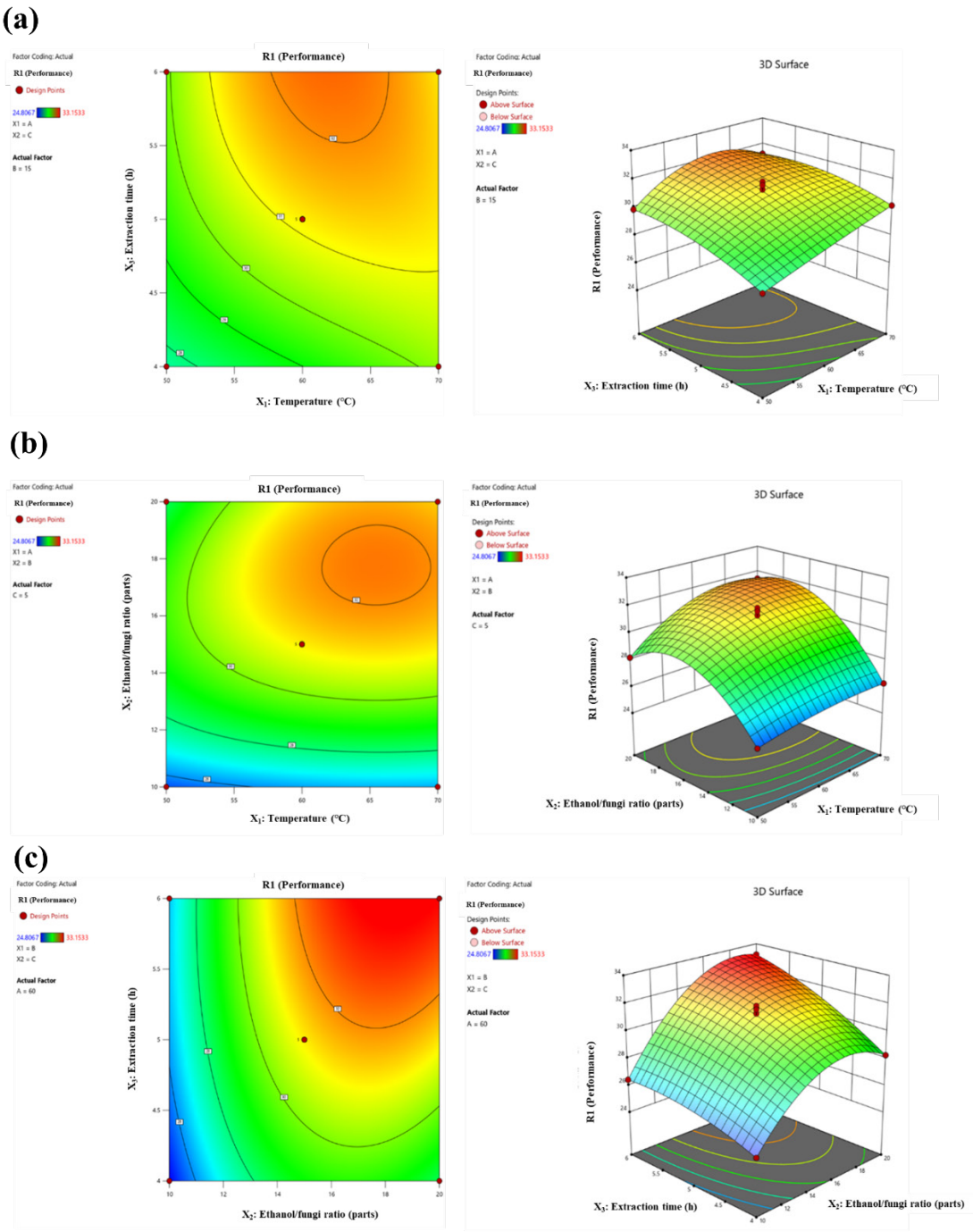

Fig. 5. 2D and 3D visualization results show the interaction between candidate variables and desired values. (a) the relationship between extraction time and temperature; (b) the relationship between ethanol/fungi ratio and temperature; and (c) the relationship between extraction time and ethanol/fungi ratio.

DISCUSSION

C. militaris is a highly valuable fungus in terms of both nutrition and medicinal properties. Cordycepin and adenosine are considered the major bioactive components, playing important roles in promoting human health (Du *et al*., 2021; Sakao *et al*., 2024). Due to these beneficial properties, *C. militaris* has become a widely exploited species, both in the wild and through artificial cultivation for fruiting body production. Today, products derived from *C. militaris* are commonly used in daily life. Consequently, it is crucial to research to increase the amount, quality, and production of cordycepin and adenosine. In the present study, we cultivated *C. militaris* under different nutritional conditions (control, T1 & T2) to evaluate the most suitable environment for mycelial growth and fruiting body development with high levels of cordycepin and adenosine. The goal was to identify the most effective cultivation process, thereby improving the quality and potency of *C. militaris*-derived preparations and facilitating future research on this medicinal fungus.

The results showed that the T1 group exhibited the highest cultivation efficiency of *C. militaris* among the three experimental

groups (control, T1 & T2), as reflected in superior mycelial growth, fruiting body development, accumulation of bioactive compounds (cordycepin & adenosine), and extraction yield. Nutrient supplementation accelerated early mycelial development (Figure 1). By day 7, T1 formed dense, cotton-like mycelia, while the control remained thin and uniform, and T2 showed intermediate growth. This suggests that organic nitrogen and carbon sources in T1 strongly promoted biomass accumulation, consistent with previous reports on soybean and carbohydrate-rich media enhancing C. militaris growth (Tang *et al*., 2018; Sripilai *et al*., 2023). Regarding fruiting body development, the T1 group showed noticeable differences compared to the control and T2 groups after 7 days (Figure 2). While mycelial color in the Control and T2 groups was uneven and primordia formation was sparse, the T1 group exhibited dense and uniform primordia growth. After 60 days, the T1 group showed significantly improved morphological indices, including fruiting body length (9.82 ± 1.44 cm), diameter (0.83 ± 0.16 cm), quantity (185.88 ± 19.53), and weight (17.18 ± 2.73 g) compared to the control and T2 groups (Figure 3). The results demonstrate that supplementing the culture medium with soybean powder and coconut water markedly promoted *C. militaris* fruiting body formation (He *et al*., 2009; Sekar *et al*., 2013; Shashidhar *et al*., 2017; Sripilai *et al*., 2023). Although both T1 and T2 were nutrient-rich media, T1 yielded significantly higher cordycepin content than T2. This difference may be explained by the nutritional composition, as T1 contained soybean powder and coconut water, which provided abundant organic nitrogen and readily available carbon sources essential for *C. militaris* growth, while T2 was supplemented only with inorganic mineral salts ($MgSO_4$, K_2SO_4). Although these salts may support the overall growth of the fungus as cofactors and osmotic stabilizers, they contribute less directly to secondary metabolic pathways. The lack of amino acids, peptides, and simple sugars in T2 may have limited the metabolic flux toward cordycepin synthesis, resulting in its lower accumulation than in T1. Sripilai *et al*. (2023) also reported that the highest cordycepin yield was achieved under conditions supplemented with 80 g/L soybean extract powder, elevating cordycepin concentration to 2.52 g/L, which exceeded that of the medium containing peptone. Similarly, Shashidhar *et al*. (2017) found that supplementing coconut water at 5% and 10% in the cultivation of *Ophiocordyceps sinensis* CS1197 increased biomass yields by 2.2- and 2.5-fold, respectively. Moreover, the antioxidative and CE-inhibitory properties of water extracts from *O. sinensis* CS1197 cultures fortified with tender coconut water and mature coconut water were higher than those of the control, suggesting a positive correlation between coconut water supplementation and enhanced antioxidant and CE-inhibitory activities. In a related study, Yuttavanichakul *et al*. (2023) showed that adding 3% coconut oil or soybean oil improved the cultivation of *C. militaris* on PDA, with colony diameters of 5.46 ± 0.15 cm and 5.66 ± 0.09 cm, respectively.

HPLC analysis revealed a significant increase in the concentration of cordycepin (337.93 ± 15.08 mg/100 g) and adenosine (394.51 ± 10.86 mg/100 g) in group T1, which were 1.98-fold and 2.38-fold higher than those in the control group, and 1.22-fold and 1.06-fold higher than in group T2, respectively. (Figure 4). Our results demonstrated higher concentrations of cordycepin and adenosine compared to those reported by Huang *et al*. (2009). The fruiting bodies of *C. militaris* cultured using rice-based, silkworm-based, and wheat-based media, obtained cordycepin content of 2.65 ± 0.02 mg/g and adenosine content of 2.45 ± 0.03 mg/g. In contrast, Kang *et al*. (2017) showed that the use of silkworm pupae resulted in the highest cordycepin content of 4.17 ± 1.66 mg/g. This could be explained by the nutritional supplementation, especially the carbon source from coconut water and the nitrogen source from soybean. Shashidhar *et al*. (2017) supplemented the culture medium with 10% (v/v) tender coconut water (TCW) and 5% (v/v) mature coconut water (MCW), respectively, and improved the adenosine and cordycepin contents by 58% and 69%, 50% and 55%. Previous studies have shown that nitrogen and carbon sources interact with each other in cordycepin production. When nitrogen sources are in excess, they will participate in the growth of mycelium, at which time carbon will play a role in energy and biomass production. On the contrary, when the input amount is sufficient, it will contribute to maintaining citric acid production (Pintado *et al*., 1998). Kang *et al*. (2014) investigated the effects of nitrogen sources using univariate experiments and Plackett-Burman methodology to identify the key factors and optimize culture conditions. This optimization led to a maximum cordycepin yield of 2008.48 mg/L in a cultivation volume of 700 mL within a 1000 mL glass jar, with a total cordycepin amount attaining 1405.94 mg/bottle.

From the above results, T1 was selected as the main subject to evaluate the extraction efficiency with 3 factors: extraction time (hr) (P=0.0065), ethanol/fungi ratio (P=0.0002), and extraction temperature (°C) (P=0.0184) (Table 3). Box-Behnken model analysis showed that the ethanol/fungi ratio factor had the strongest influence. Experiment 17, with an extraction time of 6 hr, ethanol/fungi ratio of 20:1, and extraction temperature of 60°C, gave an extraction efficiency of 33.15%, the highest compared to the remaining experiments (Table 2). This outcome is comparable to the earlier research conducted by Soltani *et al*. (2017), which showed that ethanol (30%), methanol (25%), ethyl acetate (22%), and hot water (23%), respectively, were additional solvents that rapidly impacted the extraction efficiency. This suggests that the solvent ratio plays a significant role in determining the biological activity and extraction efficiency of C. militaris (Soltani *et al*., 2017). Yang *et al*., (2014) used the Box-Behnken model to maximize the culture efficiency. The results of the amounts of mycelium, intracellular polysaccharide, adenosine, and mannitol were 12.19 g/L, 0.6 g/L, 61.84 mg/L, and 1.38 g/L, respectively, and the D value was 0.77. In conclusion, T1 was the optimal treatment for the highest efficiency in fruiting body development, nutrient content (cordycepin & adenosine), as well as extraction efficiency (60°C, ethanol/fungi ratio 20:1, 6 hr). The results showed the need to optimize the culture process to enhance the value of *C. militaris* - a valuable medicinal fungi with high economic value, widely studied and applied in agriculture, traditional and modern medicine (Qin *et al*., 2019; Du *et al*., 2021; Jędrejko *et al*., 2021; Sakao *et al*., 2024). From there, we proposed a cultivation process for *C. militaris* (Figure 6).

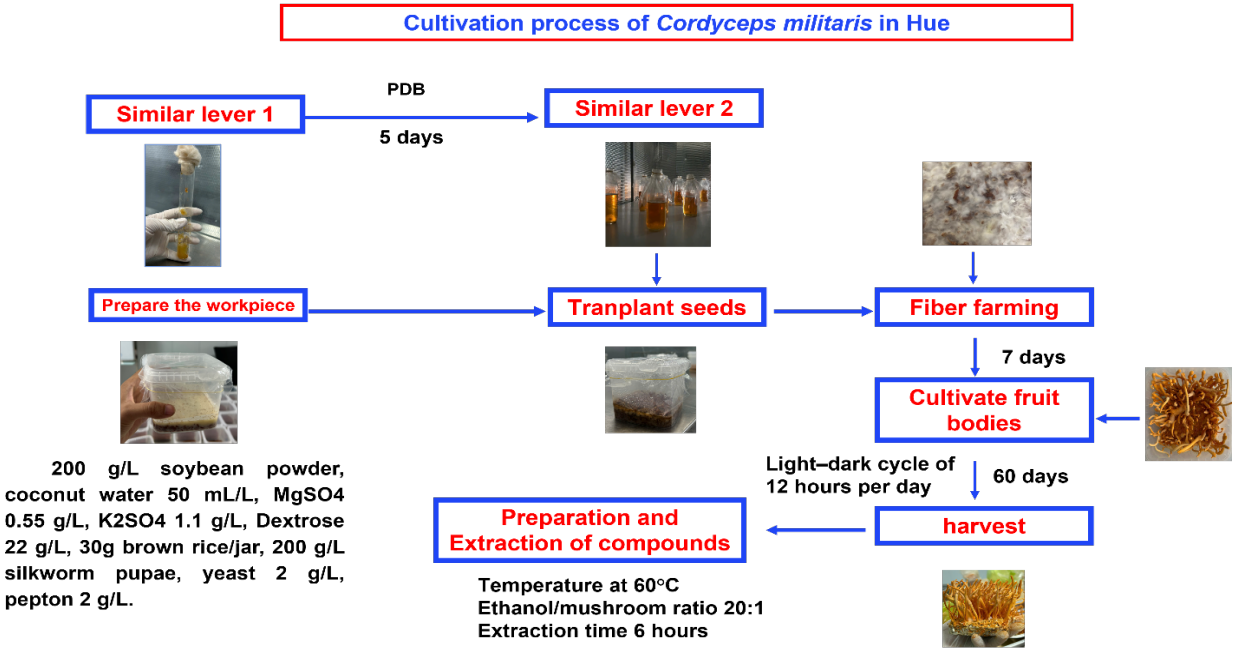

Fig. 6. Cultivation process of *Cordyceps militaris*

CONCLUSION

The study showed that the T1 group supplemented with nutritional sources such as soybean powder and coconut water as carbon and nitrogen sources improved the fruiting body parameters such as stem length (9.82 ± 1.44 cm), diameter (0.83 ± 0.16 cm), number of fruiting bodies (185.88 ± 19.53) and weight (17.18 ± 2.73 g), as well as increased the cordycepin (337.93 ± 15.08 mg/100 g) and adenosine (394.51 ± 10.86 mg/100 g) content. In order to improve the quality of *C. militaris* extraction from the results of group T1, the influencing factors: extraction temperature, extraction time, and ethanol/fungi ratio were studied within a certain range. The experiments were designed using the Box-Behnken design and subsequently optimized with Design Expert software (version 13). Under optimal conditions, the maximum yield could be increased by 33.15% with an extraction temperature of 60°C, an ethanol/fungi ratio of 20:1, and an extraction time of 6 hr. Although the results are promising, this study is limited by the narrow range of nutrient sources tested. Future studies are needed to investigate the broad spectrum of different nutrient sources affecting the quality of C. militaris as well as the molecular mechanisms of cordycepin and adenosine biosynthesis. However, this result provides a solid scientific basis for improving the large-scale cultivation and extraction of C. militaris, supporting the development of functional food, pharmaceutical, and medicinal applications.

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ETHICAL STATEMENT

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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