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Effects of carbon and nitrogen sources on fruiting body formation and cordycepin production of *Cordyceps militaris* (L.) Link

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ABSTRACT: *Cordyceps militaris* (L.) Link is an entomopathogenic fungus. It has been accumulated the bioactive compounds including cordycepin and adenosine that reduced blood cholesterol and provide improved anti-cancer, and immune support. The objectives of this study were to establish an effective medium for fruiting body formation of *C. militaris* and to evaluate cordycepin and adenosine contents. Each medium contained different types of carbon sources (sucrose, glucose) and nitrogen sources (yeast extract, peptone). For the BH strain, the highest length of fruiting bodies (4.61±0.45 cm), fresh weight (35.37 ± 1.16 g/bottle) dry weight (11.88±0.29 g/bottle) and cordycepin contents (6.92±0.09 mg/g) were observed on T6 medium supplemented with 20 g/l sucrose and 20 g/l yeast extract and the highest adenosine contents (0.10±0.02 mg/g) were found in T2 medium supplemented with 10 g/l sucrose and 10 g/l yeast extract. For the DA strain, the highest length of fruiting bodies (4.26±0.08 cm), fresh weight (31.89±0.25 g/bottle) dry weight (10.02 ± 0.15 g) and cordycepin contents (3.95±0.05 mg/g) were obtained from T6 medium supplemented with 20 g/l sucrose and 20 g/l yeast extract and the highest adenosine contents (0.72±0.00 mg/g) was achieved on T5 medium supplemented with 20 g/l glucose and 20 g/l peptone.

Keywords: *Cordyceps militaris*, carbon sources, nitrogen sources, cordycepin, adenosine

Introduction

Cordyceps militaris (L.) Link was known as the one of the Chinese traditional medicinal mushrooms and has been widely used for centuries. In natural conditions, *C. militaris* often parasitizes on the pupa or larva of insects and forms fruiting bodies. It is widely used as traditional medicinal materials and edible mushroom in China, Korea and some other Asian regions (Shrestha et al., 2012). Adenosine and cordycepin are the main bioactive components of *C. militaris* (Dworecka-Kaszak,2014; Pathania et al., 2015). Previous pharmacological studies have shown a remedial effect on a variety of diseases and condition s, with the presence of bioactive compounds has been reported to reduce blood cholesterol, to decrease the risk of cancer and protect against lipid peroxidation due to a strong antioxidant effect. Many previous reports have revealed that the fungus's biological activities studies focused on anti-cancer, anti-tumor, anti-diabetic, anti-inflammatory, immune-modulatory and sperm-improving effects (Cui, 2014; Luo et al., 2017; Wang et al., 2012). Some studies have shown its potential economically efficient fungus that can be valued highly in the context of industrialization. On the other hand, the wild fruiting bodies of *C. militaris* are difficult to find and can become invalid due to host specificity and the requirement of the strict growth environment. Due to the high pharmacological activities, *C. militaris* have recently drawn great attention in artificial culture. It is important to produce the medicinal fungus by modern culture techniques because of their pharmacological function and biological properties. The optimization of the cultivation condition is necessary for large-scale production of the fruiting bodies and cordycepin from *C. militaris*. The artificial cultivation requires the development of medium composition including carbon sources and nitrogen sources, by which the microorganism exhibits growth and in order to enhance bioactive components during ambient cultivation processes. In general, the major nutrient ingredients of medium are carbon and nitrogen sources. It is essential for cell proliferation and metabolite biosynthesis. Previous reports have demonstrated the optimization of carbon and nitrogen sources and their optimal concentrations for fruiting body formation and bioactive component production (Dang et al., 2018; Kang et al., 2017; Yang et al., 2014; Qin et al., 2018). The purposes of this research were to investigate the effects of carbon and nitrogen sources on fruiting body production and to evaluate the cordycepin and adenosine contents in different media.

Materials and methods

Materials preparation and culture conditions

Two strains of *C. militaris* (BH, DA) from Agricultural Science Program, Mahidol University Kanchanaburi Campus were used in this study. Mycelial culture was prepared on potato-dextrose-agar (PDA) medium at 20±2 ˚C for 3 weeks.

Effect of different carbon and nitrogen sources on fruiting body formation

Each of carbon sources (glucose, sucrose) was individually used at various concentrations of 10, 20 g/l. In the same way, each of nitrogen sources (yeast extract, peptone) was individually used at various concentrations of 10, 20 g/l. The medium was prepared by filling 480 ml glass bottles with 30 g of Man-pu

rice and 30 ml of modified potato medium (1 g KH_2PO_4, 0.5 g $MgSO_4$, 1 g thiamine mixed with 1,000 ml potato water which obtained from 200 g potatoes boiled in 1000 ml distilled water). After that, the medium was autoclaved at 121 ℃ for 20 min. The medium was inoculated with three mycelial discs (0.5 cm diameter) from PDA medium and was cultured at 20±2 ˚C under dark conditions for 3 weeks. Then, the medium was moved to light conditions (16 h light/ 8 h dark) until it completed the fruiting body stage for 5 weeks. The length of fruiting bodies, fresh weight, dry weight, cordycepin and adenosine contents were recorded. Culture media were prepared for treatments and denoted as follows: Control; 10g/l glucose:10g/l yeast extract, T1; 10g/l glucose:10g/l peptone, T2; 10g/l sucrose:10g/l yeast extract, T3; 10g/l sucrose:10g/l peptone, T4; 20g/l glucose:20g/l yeast extract, T5; 20g/l glucose:20g/l peptone, T6; 20g/l sucrose:20g/l yeast extract, T7; 20g/l sucrose: 20g/l peptone.

Determination of cordycepin and adenosine

Sample preparation

One gram of dried powder of *C. militaris* fruiting body was extracted with 10 ml of 50% (v/v) methanol in a 50 ml centrifuge tube. After that, the centrifuge tubes were placed in an ultrasonic cleaner at 125 watts for 30 min. The supernatant was separated by centrifugation at 9,900 rpm for 10 min and was filtered through a 0.45 µm membrane filter. The methanolic extracts of the fruiting bodies were assayed for cordycepin and adenosine contents (Huang et al., 2009).

High-performance liquid chromatography (HPLC) analysis

To measure cordycepin and adenosine, the HPLC system was equipped with a C_{18} Inertsil ODS-4 analytical column (4.6 mm x 250 mm, 5 µm particle size) (GL science Inc., Japan). The solutions were detected using a UV Visible Detector (Shimadzu LC-20A) at 254 nm. The mobile phase was performed using 85:15 (V/V) deionized water and methanol. The separation was conducted with a flow rate of 1.0 ml/min. The injection volume was 20 µl. Standards of cordycepin and adenosine were purchased from the Sigma Chemical Corporation.

Statistical analysis

The results were presented as mean±SD (n=5). All experiments were repeated for three times, with 5 culture bottles for each treatment and using completely randomized design. Statistical analyses of the results were performed at $P < 0.05$ significance level using SPSS program versions 22.0. Differences between the means of individual groups were assessed using one-way ANOVA with Duncan's multiple-range test.

Results

Effects of carbon and nitrogen sources on fruiting body formation

Effects of eight culture media on fruiting body growth of two strains were observed. The T6 medium supplemented with 20 g/l sucrose and 20 g/l yeast extract showed the highest fruiting body growth, revealing its suitability as a medium for fruiting body production in both strains. The results demonstrated that the best carbon sources was sucrose and the best nitrogen sources was yeast extract. The morphology of fruiting bodies was observed, including a yellow/orange color, cylindrical shape and a

common overall length of about 3 to 7 cm in length and growing 1 to 6 mm in width after 8 weeks of culture (**Figure 1 and 2**). The fruiting body growth of both strains on different culture medium strains on the different culture media assessed and significant differences between each treatment were found. The results showed that the average length of fruiting bodies, fresh weight and dry weight were significantly difference ($P < 0.05$) in each treatments. For the BH strain, the highest length of fruiting bodies (4.61±0.45 cm), fresh weight (35.37±1.16 g/bottle) and dry weight (11.88±0.29 g/bottle) of the BH strain were recorded on a T6 medium (20 g/l sucrose and 20 g/l yeast extract). On the other hand, *C. militaris* cultured on a T7 medium supplemented with 20 g/l sucrose and 20 g/l peptone showed the lowest length of fruiting bodies (1.57±0.32 cm). The lowest fresh weight (18.68±1.00 g/bottle) was observed on a T5 medium supplemented with 20 g/l glucose and 20 g/l peptone. In addition, the lowest dry weight (5.01±0.42 g/bottle) was observed on a T1 medium supplemented with 10 g/l glucose and 10 g/l peptone (**Table 1**).

Moreover, the results also indicated that the T6 medium was suitable for fruiting body production of the DA strain. Effects of carbon and nitrogen sources on the DA strain were indicated (**Table 2**). A high concentration of sucrose and yeast extract was optimal for fruiting body growth. The results also indicated that the highest length of fruiting bodies (4.26±0.08 cm), fresh weight (31.89±0.25 g/bottle) and dry weight (10.02±0.15 g/bottle) of DA strain fruiting bodies were occurred on the T6 medium.

Table 1 Effects of different carbon and nitrogen sources on fruiting body formation of *Cordyceps militaris* BH strain. Values represent means±SD of five replicated, Observations after 8 weeks of culture

Treatments	Length (cm)	Fresh weight (g/bottle)	Dry weight (g/bottle)
Control (10G+10Y)	2.56±0.18^{c}	29.09±1.68^{b}	10.51±0.85^{b}
T1 (10G+10P)	1.79±0.30^{d}	20.07±2.26^{d}	5.01±0.42^{f}
T2 (10S+10Y)	3.05±0.28^{c}	25.99±1.09^{bc}	9.54±0.80^{bc}
T3 (10S+10P)	1.61±0.11^{d}	22.28±1.77^{cd}	6.54±0.32^{e}
T4 (20G+20Y)	3.72±0.39^{b}	30.12±1.46^{b}	8.45±0.34^{cd}
T5 (20G+20P)	1.61±0.21^{d}	18.68±1.00^{d}	6.93±0.25^{e}
T6 (20S+20Y)	4.61±0.45^{a}	35.37±1.16^{a}	11.88±0.29^{a}
T7 (20S+20P)	1.57±0.32^{d}	19.24±0.45^{d}	7.22±0.15^{de}

* The different letters in the same column are significantly different at $P < 0.05$ according to DMRT

** The details of abbreviations; G = glucose, S = sucrose, Y = yeast extract, P = peptone

Table 2 Effects of different carbon and nitrogen sources on fruiting body formation of *Cordyceps militaris* DA strain. Values represent means ± SD of five replicated, Observations after 8 weeks of culture

Treatments	Length (cm)	Fresh weight (g/bottle)	Dry weight (g/bottle)
Control (10G+10Y)	3.55±0.20^{b}	24.25±1.44^{bc}	6.66±0.77^{bc}
T1 (10G+10P)	1.51±0.11^{c}	20.55±1.13^{cd}	6.56±0.34^{bc}
T2 (10S+10Y)	3.09±0.52^{b}	19.18±1.92^{d}	3.68±0.44^{d}
T3 (10S+10P)	1.50±0.17^{c}	25.06±0.71^{b}	7.01±0.45^{bc}
T4 (20G+20Y)	3.14±0.26^{b}	26.89±1.02^{b}	8.17±0.61^{b}
T5 (20G+20P)	1.32±0.13^{c}	19.96±1.97^{d}	5.99±1.04^{c}
T6 (20S+20Y)	4.26±0.08^{a}	31.89±0.25^{a}	10.02±0.15^{a}
T7 (20S+20P)	0.92±0.06^{c}	17.37±0.85^{d}	5.97±0.35^{c}

* The different letters in the same column are significantly different at P < 0.05 according to DMRT

** The details of abbreviations; G= glucose, S= sucrose, Y= yeast extract, P = peptone

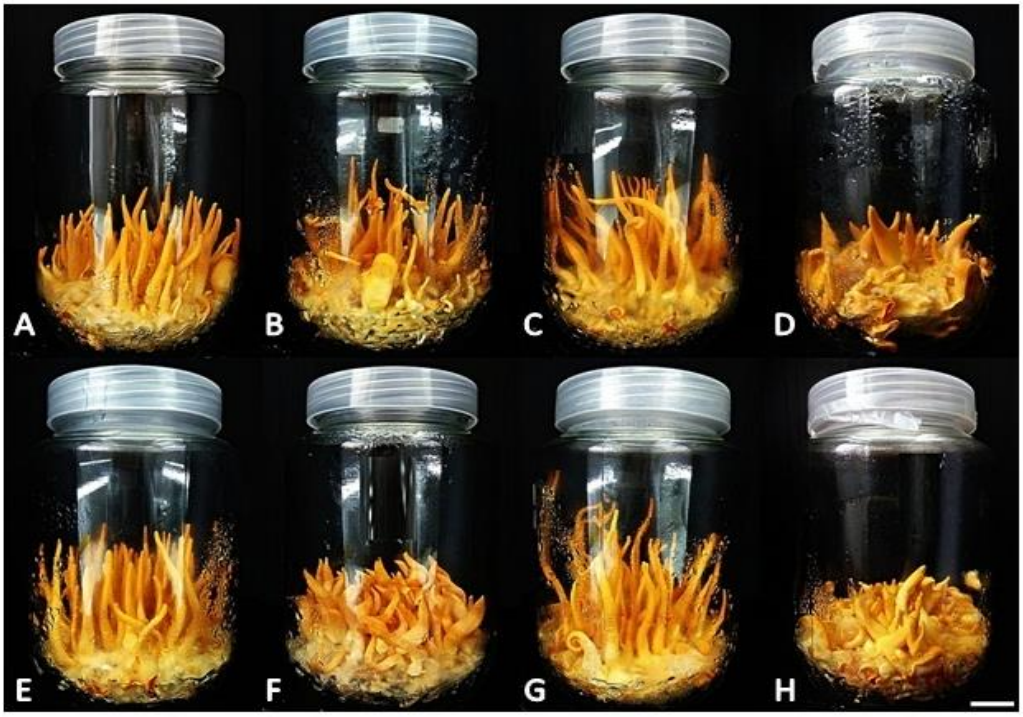

Figure 1 Fruiting bodies of *Cordyceps militaris* BH strain after 3 weeks of cultured in the dark followed by 5 weeks under light; A: Control; B: T1 medium; C: T2 medium; D: T3 medium; E: T4 medium; F: T5 medium; G: T6 medium; H: T7 medium; Scale bar= 2 cm.

Figure 2 Fruiting bodies of *Cordyceps militaris* DA strain after 3 weeks of cultured in the dark followed by 5 weeks under light; A: Control; B: T1 medium; C: T2 medium; D: T3 medium; E: T4 medium; F: T5 medium; G: T6 medium; H: T7 medium; Scale bar= 2 cm.

The cordycepin and adenosine contents

Production of cordycepin and adenosine were measured using HPLC analysis. Noticeably, the T6 medium showed a marked bioactive accumulation, and the maximum cordycepin contents of the BH strain (6.92±0.09 mg/g) while the minimum cordycepin contents (0.03±0.00 mg/g) was observed on Control supplemented with 10 g/l glucose and 10 g/l yeast extract. The results showed that the highest adenosine contents of the BH strain (0.10±0.02 mg/g) was recorded on the T2 medium supplemented with 10 g/l sucrose and 10 g/l yeast extract. In contrast, the lowest adenosine contents (0.04±0.00 mg/g) was found in Control supplemented with 10 g/l glucose and 10 g/l yeast extract (**Table 3**).

Optimal results in terms of cordycepin contents for the DA strain (3.95±0.05 mg/g) was obtained from the T6 medium whereas the T3 medium supplemented with 10 g/l sucrose and 10 g/l peptone was resulted in the lowest cordycepin contents (0.61±0.01 mg/g). Additionally, the results illustrated that the maximum adenosine contents of the DA strain (0.72±0.00 mg/g) was recorded on a T5 medium. Conversely, the lowest adenosine contents (0.05±0.00 mg/g) was found with the T7 medium (**Table 4**).

Table 3 Average of adenosine and cordycepin contents in different treatments in *Cordyceps militaris* BH strain. Values represent means±SD of five replicated, Observations after 8 weeks of culture

Treatments	cordycepin contents (mg/g)	adenosine contents (mg/g)
Control (10G+10Y)	0.03±0.00^{g}	0.04±0.00^{d}
T1 (10G+10P)	0.95±0.03^{f}	0.06±0.00^{c}
T2 (10S+10Y)	1.65±0.32^{c}	0.10±0.02^{a}
T3 (10S+10P)	0.96±0.02^{f}	0.08±0.00^{b}
T4 (20G+20Y)	1.42±0.01^{e}	0.07±0.00^{b}
T5 (20G+20P)	1.52±0.01^{d}	0.07±0.00^{b}
T6 (20S+20Y)	6.92±0.09^{a}	0.07±0.00^{b}
T7 (20S+20P)	6.06±0.03^{b}	0.07±0.00^{bc}

* The different letters in the same column are significantly different at P < 0.05 according to DMRT

** The details of abbreviations; G= glucose, S= sucrose, Y= yeast extract, P = peptone

Table 4 Average of adenosine and cordycepin contents in different treatments in *Cordyceps militaris* DA strain. Values represent means±SD of five replicated, Observations after 8 weeks of culture

Treatments	cordycepin contents (mg/g)	adenosine contents (mg/g)
Control (10G+10Y)	0.85±0.00^{f}	0.12±0.00^{c}
T1 (10G+10P)	1.87±0.00^{b}	0.28±0.01^{b}
T2 (10S+10Y)	0.98±0.01^{e}	0.11±0.00^{d}
T3 (10S+10P)	0.61±0.01^{g}	0.06±0.00^{fg}
T4 (20G+20Y)	0.83±0.00^{f}	0.08±0.00^{e}
T5 (20G+20P)	1.06±0.01^{d}	0.72±0.00^{a}
T6 (20S+20Y)	3.95±0.05^{a}	0.06±0.00^{f}
T7 (20S+20P)	1.68±0.00^{c}	0.05±0.00^{h}

* The different letters in the same column are significantly different at P < 0.05 according to DMRT

** The details of abbreviations; G= glucose, S= sucrose, Y= yeast extract, P = peptone

Discussion

The major nutrients ingredients in the media were carbon and nitrogen sources. Several factors can affect successful cultivation, including the strain used, culture temperature, light intensity, and chemical substances in the culture media. Carbon is the most important nutrient required for fruiting body growth. Glucose and sucrose are often used as carbon sources for increase fruiting body production. Nitrogen sources are also essential nutrients for fungal growth (Das et al., 2010). Various nitrogen sources including yeast extract and peptone were used in this study. The results showed that there were significant differences in fruiting body length, fresh weight, dry weight, adenosine contents and cordycepin

contents according to the various treatments used. In all eight treatments, sucrose and yeast extract were favorable nutrients for fruiting body formation. *C. militaris* is preferred sucrose and yeast extract as the best carbon and nitrogen sources. Vigorous fruiting bodies were produced using the optimized medium. Sucrose is a disaccharides and is the most common carbohydrate used as a carbon source in artificial cultures, while glucose is a monosaccharides that is easier to absorb than sucrose. However, sucrose influences cell enlargement by maintaining osmotic pressure in the fungal cells that increases the fruiting bodies growth (Neto and Otoni, 2003). Previous study was reported the growth characteristics of *C. militaris* cultivated on sucrose, glucose and xylose. Due to its maximum growth rate and biomass productivity, sucrose is the preferred carbon sources for the growth of fungal cells by showing the maximum growth rate and the highest biomass productivity. The individual molecules of glucose and fructose may interact with the central carbon metabolism as needed for biomass formation, leading to enhanced biomass production in the sucrose culture (Raethong et al., 2018).

Moreover, nitrogen is an essential requirement for growth, and the ability to metabolize. A wide variety of nitrogen sources, but most importantly yeast extract, is required for fungal viability. Previous report have revealed that yeast extract containing 30% protein, 0.42% fat, 9.2% total volatile nitrogen and other soluble components provides plenty of nutritional sources for fungal culture. (Zarei et al., 2016). The results of the present study are similar to those in several other studies that show the growth of fruiting bodies was greatly influenced by the composition of the culture media. Interestingly, previous studies examined the effects of carbon sources (glucose, sucrose) and nitrogen sources (yeast extract, peptone and milk powder) on the production of fruiting bodies were examined. The best fruiting body production (2.77±0.9 g/bottle) was found using Man-pu rice supplemented with 5 g/l sucrose and 30 g/l milk powder (Ritdate et al., 2015). The medium composition and conditions of the submerged culture of *C. militaris* were studied. The optimal medium supplemented with 10.33 g/l yeast extract, 27.24 g/l sucrose, and 5.60 g/l KH_2PO_4 were recorded (Yang et al., 2014). In addition, Singpoonga et al. (2016) reported that a yeast potato dextrose medium supplemented with sucrose, beef extract, zinc chloride and folic acid was produced the maximum mycelial yield. The highest fresh weight (40.98 g/bottle) was found using a 50 g rice medium supplemented with 40 ml modified of potato dextrose broth, 30 g silkworms and 10 ml whole eggs. The highest adenosine (125.98 mg/100g DW) and cordycepin contents (479.93 mg/100g DW) were also determined. Additionally, the artificial cultivation on purple brown rice, jasmine brown rice, and black jasmine rice has been investigated. The highest mycelium density was found using black jasmine rice supplemented with 15 g glucose, 10 g peptone, 10 g yeast extract, 1 g KH_2PO_4, 0.5 g $MgSO_4$ and 0.5 g thiamine mixed in 1,000 ml potato water (Sornprasert et al., 2012; Sornprasert and Hambananda, 2016). Thus, the concentrations of carbon and nitrogen sources in culture media are influential factors in fruiting body production.

Conclusions

The effects of culture medium composition including carbon and nitrogen sources on the growth of fruiting bodies were investigated. *C. militaris* was inoculated on potato dextrose agar and cultivated in an artificial medium with various carbon (sucrose and glucose) and nitrogen (yeast extract and peptone) supplements. The different carbon and nitrogen sources showed different effects on fungal growth. The results indicated that the medium supplemented with 20 g/l sucrose and 20 g/l yeast extract (T6) was the most suitable medium for fruiting body production. In addition, the production of cordycepin and adenosine production through the various treatments were measured using HPLC.

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