OPTIMAL PRODUCTION OF CORDYCEPIN AND ADENOSINE FROM *CORDYCEPS CICADAE* BG01 AND IMPACTS OF THE TOTAL EXTRACT ON BLOOD BIOCHEMICAL OF SWISS WHITE MICE

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SUMMARY

Cordyceps cicadae (*C. cicadae*) is one of the most valued medicinal mushrooms since it has been found containing precious bioactive compounds that help improve human health, prevent cancer, etc. In this study, we have optimized the major factors affecting the biosynthesis of cordycepin and adenosine in *C. cicadae* fruiting bodies and evaluated the impacts of the liquid extracted from *C. cicadae* BG01 on Swiss white mice. The optimal cultivation conditions for the biosynthesis of cordycepin and adenosine in *C. cicadae* fruiting body have been proved to include MT2 medium, temperature of 20°C, light intensity of 500 lux (the lighting cycle of 12h/12h) and fruiting bodies harvested after 50 days. Under these conditions, the content of cordycepin and adenosine in *C. cicadae* fruiting body were 2,72 mg/g and 3,23 mg/g, relatively. Simultaneously, the effects of the liquid extracted from of *C. cicadae* BG01 on the blood biochemical indices of Swiss white mice were also determined. As stated in, the liquid extracted from *C. cicadae* BG01 fruiting body neither causes toxicity nor damages to liver and kidney functions of mice at the dose of 250 µL/mouse/day equivalent to 780 mg dried fungus/kg of body weight for 4 weeks.

Keywords: Adenosine, cordycepin, *Cordyceps cicadae*, fruiting body, swiss white mice.

INTRODUCTION

In traditional Chinese medicine, *C. cicadae* has been regarded as one of the most precious herbs with the value equivalent to *C. sinensis*. The main bioactive ingredients that have been identified in the fungus *C. cicadae* including ISP-1 (myriocin), adenosine, cordycepin, N6- (2-hydroxyethyl) adenosine (HEA), ergosterol, peroxide, cyclic heptapeptide, polysaccharides… (Hsu *et al*., 2015). In particular, cordycepin and adenosine are two widely known compounds considered to be effectively anti-cancer. Due to containing all these components, *C. cicadae* has plenty of scientifically proven effects such as anti-tumor activities, kidney functions recovery, health improvement (Wang *et al*., 2001; Zhu *et al*., 2011).

Recently in Vietnam, researches and cultivations of *Cordyceps* fungi have been strongly developed. However, the species cultivated is mostly *C. militaris* and almost all strains being produced are imported from abroad. Until now, there has been no official research on optimizing the factors related to the biosynthesis of cordycepin and adenosine, and the effects of those on animal models of *C. cicadae* fruiting body cultivated in Vietnam.

The factors such as the nutrient ingredients in the cultivation media, the cultivation temperature and the light intensity are the ones which do not only directly influence the formation and development of the fungus fruiting body but also affect the biosynthesis of different bioactive compounds in the fungus. In addition, the duration of cultivation can also affect the quality of the fruiting body. On time harvesting time the fruiting bodies would help to obtain higher quality and content of bioactive components. If they are harvested before the due time, the fruiting bodies would not have fully developed yet and the bioactive components also have not accumulated enough, leading to the low quality of these fruiting bodies. In contrast, if they are harvested late, the fungus fruiting body would be dehydrated and become shaggy, resulting in ugly hyphae and decreased quality. In this research, we have optimized the major factors for production and the biosynthesis of cordycepin and adenosine in the fruiting body of *C. cicadae* BG01 (the strain isolated in Vietnam and successfully cultivated by the research group) and the impacts of the liquid extracted from its fruiting body on Swiss white mice.

MATERIALS AND METHODS

Materials

C. cicadae strain BG01 isolated in Vietnam which has been introduced in the previous publication of our research group (Binh *et al*., 2017). Isolated strains are maintained at Genomics Unit, National Key Laboratory of Enzyme and Protein Technology, VNU University of Science.

4-weeks-old male Swiss white mice were provided by the National Institute of Hygiene and Epidemiology, all mice were healthy and qualified during experiments.

Methods

Optimizing the major factors affecting the content of cordycepin and adenosine

The strain *C. cicadae* BG01 was cultivated as reported by Duong Nghia Binh *et al*. (2017) with some modifications. Fungi fruiting bodies were collected from different cultivation conditions, and measured the content of adenosine and cordycepin by the high performance liquid chromatography (HPLC) system. The factors were evaluated including:

a) Nutrient ingredients experiments were conducted in three nutritional media: MT1: 20g white rice, 5g powder of fresh silkworm cocoon; MT2: 20g unpolished rice, 5g powder of fresh silkworm cocoon; MT3: 20g white rice, 5g powder of the *Brihaspa atrostigmella* larva; MT4: 20g unpolished rice, 5g powder of the *Brihaspa atrostigmella* larva, 50ml of nutritional fluid was then added to each type of medium listed. The components of 1 liter of nutritional fluid consist of the extract fluid from 200g potato, 20g glucose, 1g peptone;

b) Temperature for fruiting bodies cultivation: experiments of fungi cultivation were performed at different temperatures: 18^{o}C, 20^{o}C, 25^{o}C;

c) Light intensity: experiments of fungi cultivation were performed at different lighting conditions with the intensity of 200 lux, 500 lux and 1000 lux (with lighting cycle 12 hours lighting/ 12 hours in darkness).

d) Ccultivation duration: after different period of cultivation (40, 50, 60 and 70 days), fungi fruiting bodies were collected and dried, then used for adenosine and cordycepin content analysis.

Evaluating the impacts of C. cicadae BG01 mushroom extract liquid on the blood biochemical indices of Swiss white mice

The mushroom extract liquid was obtained by method of Jung *et al*. (2004): 6g of *C. cicadae* fruiting bodies were freeze dried, grounded and boiled in a bain-marie with water (extracted by water) or with 30% alcohol (extracted by alcohol) at the temperature of 70^{o}C in 20 hours. The mushroom extract liquid was then adjusted to 100ml per sample.

Experiment design: experiments were performed with 3 different mice groups and repeated independently three times. The Swiss white mice were divided into 3 groups, each group contained 5 individuals, the experimental mice at the beginning had the average weight of 19g/individual. Group 1: the mice were fed with the mushroom liquid extracted by water. Group 2: the mice were fed with the mushroom liquid extracted by alcohol. Group 3: Control group - the mice were fed with sterile water.

The mice in each group were raised with the same diet and monitored daily. The oral dose of the fruiting bodies extract liquid applied on the mice in group 1 and group 2 were 250 µL/mouse/day (equivalent to approximately 780 mg of dried fungus/kg of body weight). The control group was fed with sterile water in 4 continuous weeks. Mice in all formulas were monitored and recorded for their expression in morphology, vitality and weight increase throughout the experiment. After 4 weeks, the mice's blood in each group were taken for analysis of their serum biochemical indices such as erythrocytes, leukocytes, platelets, hemoglobin, hematocrit, creatinine, GOT, GPT, ect. Blood samples collection and analysis were conducted at the Department of Biochemistry, Hospital of Textile.

RESULTS AND DISCUSION

Effects of the major factors on content of cordycepin and adenosine

The content of adenosine and cordycepin are two of the most important criteria for evaluating the quality of the fruiting body of the Cordyceps genera (Lu *et al*., 2015). Firstly, we have evaluated the influences of some major factors on the content of adenosine and cordycepin from fungus *C. cicadae* BG01. In fact, during harvesting, there are two parts of the fungus, the fruiting body, and the substrate containing a lot of hyphae. Determining the content of cordycepin and adenosine in both of the fruiting body and the cultivation substrate will help guide the harvesting of this fungus in real life production. After 50 days of culture under the introduced conditions (Binh *et al*., 2017), we proceeded to collect the fungus fruiting body and the substrate. Both the fruiting body and the substrate were then dried on a freeze-dryer system until the moisture reached 10%. The analysis result showed that the content of adenosine and cordycepin in the fruiting body was much higher than in the substrate containing hyphae (Figure 1). The fruiting body may be the part containing much bioactive components and could be valuable. Therefore, in the subsequent studies, we performed evaluating the effects of cultivation factors through the content of cordycepin and adenosine in the fungus fruiting body.

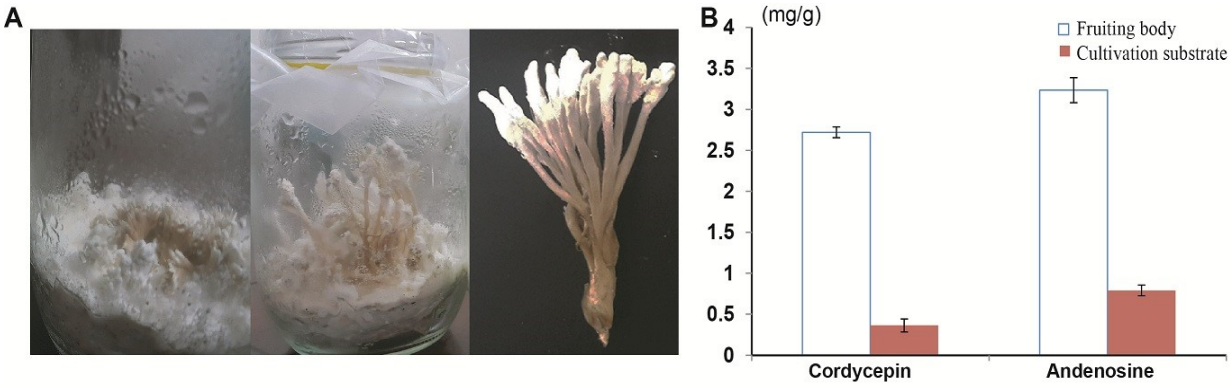

Figure 1. Content of adenosine and cordycepin in the fruiting body and the cultivation substrate of *C. cicadae* BG01

(A) Fungus fruiting body and the cultivation substrate; (B) Content of adenosine and cordycepin

We evaluated effects of several factors including the nutrient ingredients in the cultivation media, the cultivation temperature, the light intensity and the duration of cultivation on the content of adenosine and cordycepin in the fruiting body of *C. cicadae* BG01.

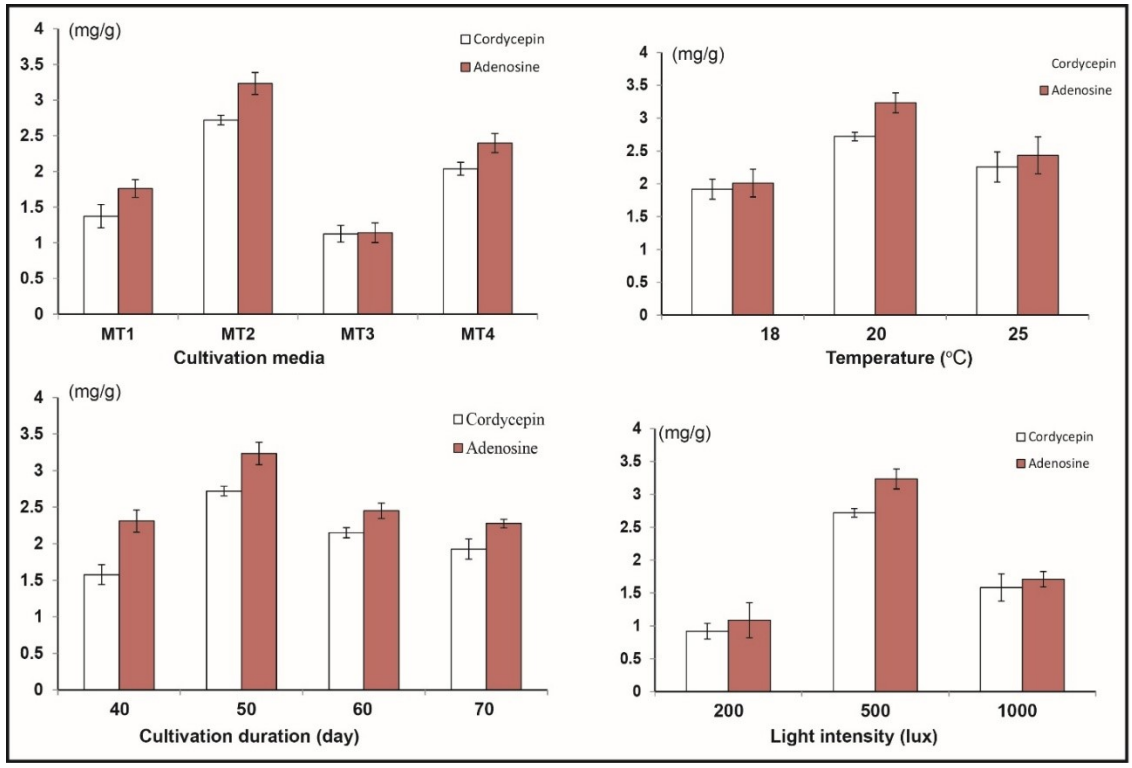

Figure 2. Impacts of several factors on the content of cordycepin and adenosine of the fungus fruiting body

The results showed that all these mentioned factors had effects on the biosynthesis of adenosine and cordycepin inside the fruiting body. With the cultivation media MT2, at the temperature of 20°C, light intensity of 500 lux (lighting 12 hours/day) and harvesting after 50 days of growth, the fungus fruiting body obtained the highest content of adenosine and cordycepin, which was 3.23 mg/g and 2.72 mg/g, relatively (Figure 2). The content of adenosine and cordycepin of fruiting body in this research is consistent with results of Li *et al*. (2007) and Lu *et al*. (2019). However, in comparison with the content of adenosine, and cordycepin of the fruiting body sample from the strain *C. cicadae* MP12 cultivated in China, Wang *et al*. (2012), our results are 2.27 times and 1.95 times higher, respectively. In comparison with other species from the same genera *Cordyceps*, the content of cordycepin in *C. cicadae* fruiting body in this study is also higher than in *C. militaris* (that is 2.654 mg/g), and especially 2.77 times higher than in the fruiting body of the natural strain of *C. sinensis* (that is 0.9801 mg/g)

cultivated in China (Huang *et al*., 2009). In conclusion, we have initially identified the optimal cultivation conditions which will propose a potential source of high-quality *C. cicadae* fruiting body in the future.

Effects of fungus fruiting body extract liquid on the blood biochemical indices of Swiss white mice

During the process of the experiments, all mice showed normal expression in morphology, behaviors in eating, drinking and other activities. After 4 weeks of applying the mushroom extract liquid on mice, it was recorded that all mice in different experiments lived healthily. This proven that the oral dose of 250 µL/mouse/day which was equivalent to approximately 780 mg of dried *C. cicade*/kg of body weight was safe to Swiss white mice. Thus, fungus *C. cicadae* does not cause acute toxicity and is safe towards mice when used at doses of 780 mg dried fungus/kg of body weight. These results are completely consistent with the study of Holliday and Cleaver (2008) which proved that *Cordyceps* fungi have no toxin with wide threshold (Holliday, Cleaver., 2008).

To give a more accurate evaluation about the effects of *C. cicadae* extract liquid on the experimental mice, we studied the changes in their blood biochemical indices. The analysis results of mice from the control group (group 3) and experimental groups (group 1 and 2) were taken after 4 weeks of experiments, represented in Table 1. We then used the T-test function to evaluate the differences between the control group and the experimental groups in terms of the blood biochemical indices, and the results demonstrated that these differences were not statistically significant ($p>0.05$). This means that there was no sign of toxicity in the mice fed with the *C. cicadae* extract liquid at the oral dose of 250 µL/mouse/day equivalent to approximately 780 mg of dried *C. cicadae*/kg of body weight. The results obtained from the control group were similar to those published by different authors (Wirth *et al*., 2009). However, there has been no report about the effects of *C. cicadae* extract liquid on mice blood biochemical indices before.

Table 1. Blood biochemical indices of mice in three experiment groups

Index	Unit	Experiment groups				
		Group 1 (extracted by water)		Group 2 (Extracted by alcohol)		Group 3 (Control)
			p-Control		p-Control	
WBC	10^9/L	5,57 ± 0,32	> 0,05	5,2 ± 0,10	> 0,05	5,7 ± 0,2
RBC	10^{12}/L	7,84 ± 1,35	> 0,05	7,66 ± 1,69	> 0,05	7,98 ± 0,28
HGB	g/L	99 ± 22,71	> 0,05	100,67 ± 28,43	> 0,05	110,67 ± 4,16
HCT	L/L	0,33 ± 0,08	> 0,05	0,32 ± 0,09	> 0,05	0,34 ± 0,01
PLT	10^9/L	434,33 ± 92,14	> 0,05	430,67 ± 31,56	> 0,05	433,67 ± 10,60

WBC: White blood cells; RBC: Red blood cells; HGB: Hemoglobin; HCT: Hematocrit; PLT: Platelets

Changes in the function of liver and kidney after 4 weeks affected by the mushroom extract liquid were determined through the indices of enzyme glutamic oxaloacetic transaminase (GOT), glutamate-pyruvate transaminase (GPT) and creatinine (Table 2). We then used the T-test function to compare the indices of GOT, GPT and creatinine between the control group and the experimental groups, the results showed that the differences among the indices are not statistically significant ($p>0.05$). Thus, the *C. cicadae* extract liquid does not cause damage to liver and kidney function of the mice at the oral dose of 250 µL/mouse/day equivalent to approximately 780 mg of dried *C. cicadae*/kg of body weight in 4 continuous weeks.

Table 2. Biochemical indices of liver and kidney of the mice in three experiment groups

Index	Unit	Experiment groups				
		Group 1 (extracted by water)		Group 2 (Extracted by alcohol)		Group 3 (Control)
			p-Control		p-Control	
Creatinin	umol/L	19 ± 3,47	> 0,05	21,33 ± 3,06	> 0,05	22 ± 1,73
AST (GOT)	U/L	223,33 ± 145,74	> 0,05	237 ± 47,51	> 0,05	227,67 ± 35,44
ALT (GPT)	U/L	50,33 ± 14,05	> 0,05	49,33 ± 9,29	> 0,05	50,67 ± 7,77

CONCLUSION

We have successfully optimized the major cultivation factors affecting the biosynthesis of cordycepin and adenosine in the fruiting body of *C. cicadae* BG01 which include the culture medium MT2, the temperature of 20°C, the light intensity of 500 lux (lighting 12 hours/day) and harvesting after 50 days of cultivation.

The *C. cicadae* extract liquid is safe for Swiss white mice at the oral dose of 250 µL/mouse/day (equivalent to approximately 780 mg dried fungus/kg of body weight) in 4 continuous weeks of usage.

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TỐI ƯU ĐIỀU KIỆN SINH TỔNG HỢP CORDYCEPIN, ADENOSINE Ở *Cordyceps cicadae* BG01 VÀ TÁC ĐỘNG CỦA DỊCH CHIẾT TỔNG SỐ LÊN CHỈ SỐ SINH HÓA MÁU CHUỘT NHẮT TRẮNG SWISS

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TÓM TẮT

Nấm *Cordyceps cicadae* là một trong những loại nấm dược liệu có chứa nhiều loại hợp chất có giá trị giúp nâng cao sức khỏe cho con người, phòng chống ung thư... Trong nghiên cứu này chúng tôi đã tối ưu được một số yếu tố chính ảnh hưởng tới sự sinh tổng hợp cordycepin, adenosine trong quả thể nấm *C. cicadae* BG01 và đánh giá được tác động của dịch chiết quả thể nấm lên chuột nhắt trắng Swiss. Điều kiện tối ưu cho quá trình sinh tổng hợp cordycepin và adenosine trong quả thể nấm là: môi trường MT2, nhiệt độ 20°C, cường độ chiếu sáng 500 lux (chiếu sáng 12h/ngày) và thời gian thu quả thể nấm là sau 50 ngày nuôi cấy. Ở điều kiện tối ưu hàm lượng cordycepin, adenosine trong quả thể nấm tương ứng đạt 2,72 mg/g và 3,23 mg/g. Đồng thời, trong nghiên cứu này tác động của dịch chiết quả thể nấm lên chỉ số sinh hóa máu chuột nhắt trắng swiss cũng được xác định. Kết quả nghiên cứu về sinh hóa máu cho thấy, dịch chiết quả thể nấm *C. cicadae* BG01 không gây ngộ độc cũng như tổn thương về chức năng gan và thận cho chuột với liều uống 250 µl/chuột/ngày tương đương khoảng 780 mg nấm *C. cicadae*/kg thể trọng trong 4 tuần liên tiếp.

Từ khóa: adenosine, cordycepin, *Cordyceps cicadae,* chuột nhắt trắng swiss, quả thể nấm.

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