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Determination of Adenosine, Cordycepin and Ergosterol Contents in Cultivated *Antrodia camphorata* by HPLC Method

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ABSTRACT

The concentrations of adenosine and cordycepin, 3'-deoxyadenosine in the hot water extract and ergosterol in the ethanol extract of a cultivated *Antrodia camphorata* were measured by high performance liquid chromatography (HPLC) method. The procedure were carried out on a reversed-phase C18 column by linear gradient elution, using four eluents as mobile phase and by isocratic elution, using 100% methanol as mobile phase, respectively. The analysis was completed within 20 min. The application of this method for quantitation of adenosine and cordycepin in 5-L and 100-L fermentation from two *A. camphorata* strains BCRC 35396 and BCRC 35398 were described. Comparison of adenosine, cordycepin and ergosterol contents were made with different treatment of 700-L fermentation broth.

Key words: adenosine, cordycepin, ergosterol, *Antrodia camphorata*, high performance liquid chromatography (HPLC) method

INTRODUCTION

Antrodia camphorata, a native species in Taiwan, grows naturally on *Cinnamomum kanehirai* Hay, a kind camphor tree native to Taiwan only, and is well known as "Niu-chang-chih". This fungus forms fruit bodies that causes brown rot on *C. kanehirai*[(1-3)]. Aborigines living in Taiwan's mountain ranges have used this fungus as traditional herb for the treatment of food and drug intoxications, diarrhea, abnormal pains, hypertension and liver cancer as Taiwan folk medicine[(2,4)]. The tonic nature of many fungi was also thought to be attributed to the cardiovascular effects of several nucleoside analogues, including adenosine and cordycepin[(1,2,4-17)]. The current price of fresh fruiting bodies of *A. camphorata* is estimated to be US$ 1500 per kg.

Cordyceps sinensis, a well known medical fungus, has long been used as Chinese folk medicine for a thousand years. Among the two effective bioactive compounds, adenosine induces apoptosis in human leukemia cell and cordycepin is a cytotoxic compound which interferes the synthesis of RNA and DNA[(6)]. The existence of adenosine and cordycepin in *A. camphorata* was never reported. Ergosterol is the dominant sterol in the membranes of higher fungi[(18,19)]. Ergosterol is rapidly degraded upon cell death and hence is a potential marker of viable biomass. Several studies have measured the ergosterol contents from ectomycorrhizal fungi[(19,20)]. Recently, the technique of ergosterol analysis has gained favor in ecological studies concerned with quantification of viable fungal biomass[(21,22)]. The objective of this study is to establish a rapid extraction and rapid analysis procedure of adenosine, cordycepin and ergosterol from different strains of cultivated *A. camphorata* mycelia.

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MATERIALS AND METHODS

I. *Chemicals*

Adenosine, cordycepin (3'-deoxyadenosine) and ergosterol (5,7,22-ergostatrien-3ß-ol; provitamin D) were purchased from Sigma (St. Louis, MO, USA). Other chemicals were analytical grade.

II. *Strain and Culture Condition*

(I) *Organism*

Strains of *A. camphorata* (BCRC 35396 and BCRC 35398) were purchased from the Bioresources Collection & Research Center. (FIRDI, Hsinchu, Taiwan, ROC).

(II) *Inoculum Preparation*

A. camphorata strains BCRC 35396 and BCRC 35398 were cultured and maintained at 26°C on M25 agar plate (Merck, Germany). Conidiospores obtained from the edge of 21-day-old colonies were used as the inoculum source.

(III) *Culture Conditions*

The experiments were carried out in 5-L fermentors (Modular Microferm Bench Top Fermentor, Model BIOFLO

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3000, New Brunswick Scientific Co., Inc., New Jersey, USA) at 28°C. The cultured media of *A. camphorata* strains are shown as the following: glucose 3%, bacto-peptone 1.5%, yeast extract 1.5%, malt extract 1.5%, $MgSO_4 \cdot 7H_2O$ 0.03%, KH_2PO_4 0.03% and K_2HPO_4 0.03%, pH 5.5. The entire fermentor with 3-L of culture medium was autoclaved at 121°C for 30 min. Three hundred milliliters of 7-day-old shake flask with the same medium were used as inoculum to each fermentor. The inoculum size was 9% (v/v) of the final volume. The fermentation conditions were set at agitation of 150 rpm and aeration rate of 1 vvm. The experiments were further scale-up to 100-L and 700-L fermentor respectively. The culture conditions of 100-L fermentor were as follows: working volume: 60-L; temperature: 28°C; agitation: 70 rpm; aeration: 1 vvm; back pressure: 0.2 kg/cm^2. The culture conditions of 700-L fermentor were as follows: working volume: 400 -L; temperature: 28°C; agitation: 60~70 rpm; aeration: 1~1.2 vvm; back pressure: 0.3 kg/cm^2.

III. *Cell Mass Determination*

Cell mass was determined by measuring the dry cell weight of 10 g suspension taken from the fermentor. The mycelia were collected by centrifugation at 5,200 ×g for 20 min. Cells were dried at 105°C to constant weight.

IV. *Extraction and Analysis of Adenosine and Cordycepin from Antrodia camphorata Strains*

One gram of dry cultivated mycelia of *A. camphorata* were ground into powder and extracted with distilled water (10 w/v)[13,23,24] followed by incubation in 100°C water bath for 1 hr. The hot water extract was separated from cells by centrifugation at 20,200 ×g for 10 min. The supernatant was then filtered through 0.45 μm membrane filter and stored at 4°C. The HPLC system was consisted of Waters Model Alliance 2690 and 996 photodiode array detector set at 254 nm wavelength (Waters, USA). The separations were achieved with a 15-μL loop, and a reversed-phase column (Merck LiChrospher 100 RP-18, 5 μm, 4.0 × 250 mm I.D.; Darmstadt, Germany) followed by linear gradient elution using eluents A, B, C and D [A: H_2O; B: CH_3CN/MeOH (1:1, v/v); C: 0.1 N HCl; D: 0.1 N $NH_4H_2PO_4$ (adjusted to pH 4.0 with H_3PO_4)] according to the following A-D profile: 0~15 min, 60~30% A, 0~30% B, 20% C, 20% D; 15~20 min, 30~60% A, 30~0% B, 20% C, 20% D. The gradient started with 60% solvent A, 0% solvent B, 20% solvent C and 20% solvent D and was linearly decreased to 30% solvent A, increased to 30% solvent B over 15 min. Afterwards, the gradient linearly increased to 60% solvent A and decreased to 0% solvent B in 15~20 min. It was finally remained at 60% solvent A, 0% solvent B, 20% solvent C and 20% solvent D for another 5 min. The flow rate was kept at 0.8 mL/min.

V. *Standard Solution of Adenosine and Cordycepin*

Standard solutions of adenosine were prepared into 0.040, 0.067, 0.100 and 0.400 mg/mL by diluting with distilled water from 4 mg/mL stock. Standard solutions of cordycepin were prepared into 0.010, 0.016, 0.024 and 0.096 mg/mL by diluting with distilled water from 0.960 mg/mL stock. The standard curve was calibrated by plotting the peak area vs. concentration of each standard after HPLC analyses as described above.

VI. *Extraction and Analysis of Ergosterol from Antrodia camphorata Strains*

Two grams of dry cultivated mycelia of *A. camphorata* were ground into powder and extracted with ethanol[19,22,25] followed by incubation in 50°C water bath for 1 hr. The hot solvent extract was separated from cells by centrifugation at 20,200 ×g for 10 min. The supernatant was then filtered through 0.45 μm membrane filter and was stored at 4°C. The HPLC system was consisted of Waters Model Alliance 2690 and 996 photodiode array detector set at 271 nm wavelength (Waters, USA). The separations were achieved with a 15-μL loop, a reversed-phase column (Merck LiChrospher 100 RP-18, 5 μm, 4.0 × 250 mm I.D.; Darmstadt, Germany) and by isocratic elution, using 100% methanol as mobile phase. The flow rate of eluent is 1.0 mL/min.

VII. *Standard Solution of Ergosterol*

Standard solutions of ergosterol were prepared into 0.1, 0.2, 0.3 and 0.4 and 0.5 mg/mL by diluting with ethanol from 1 mg/mL stock. The standard curve was calibrated by plotting the peak area vs. concentration of each standard after HPLC analyses as described above.

VIII. *Homogenation and Spray-drying from 700-L Fermentation Broth*

The cultured broth of 700-L fermentor with solid content about 13% (w/v) was homogenized and sent to the spray-dryer without carrier. The operating condition of spray-drying was as follows: fluid pressure of nozzle at 2.8 kg/cm^2. The inlet and outlet temperature of the spray-dryer were reached 192°C and 104°C, respectively.

RESULTS AND DISCUSSION

I. *Chromatograms of Adenosine and Cordycepin*

The chromatogram of standard adenosine and cordycepin is shown in Figure 1. The chromatogram of sample analyzed is shown in Figure 2. The retention times of adenosine and cordycepin for the sample were within 10 min. Compared with other methods, this method is rapid, accurate, and highly reproducible[26,27,28]. Shiao *et al.*[28] reported the profiles of nucleosides and nitrogen

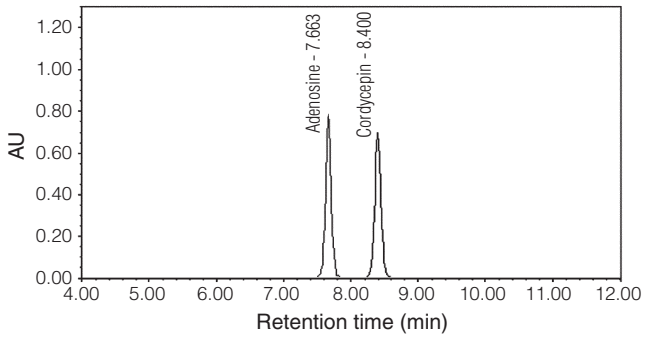

Figure 1. Chromatogram of standard adenosine and cordycepin.

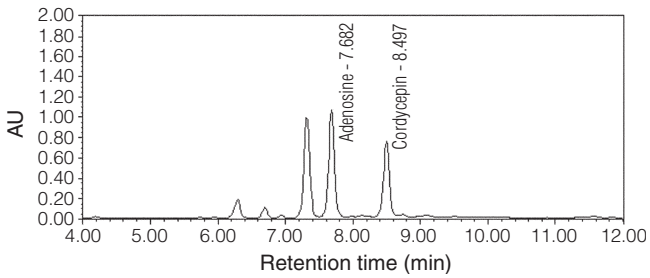

Figure 2. Chromatogram of sample.

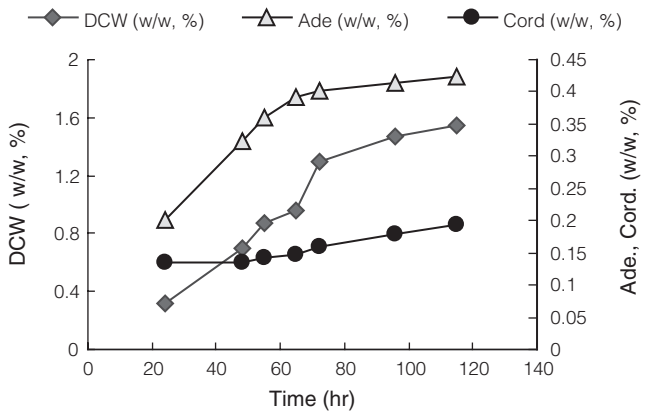

Figure 3. Time course of *A. camphorata* strain BCRC 35396 cultivated in a 5-L fermentor. DCW: dry cell weight (w/w, %); Ade: Adenosine (w/w, %); Cord: cordycepin (w/w, %).

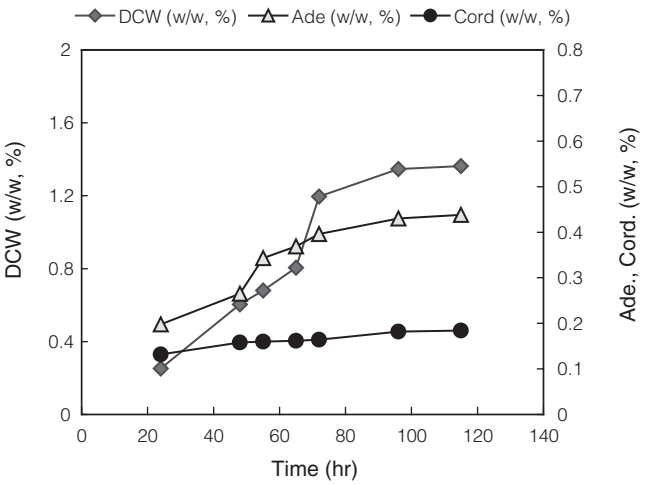

Figure 4. Time course of *A. camphorata* strain BCRC 35398 cultivation in a 5-L fermentor. DCW: dry cell weight (w/w, %); Ade: Adenosine (w/w, %); Cord: cordycepin (w/w, %).

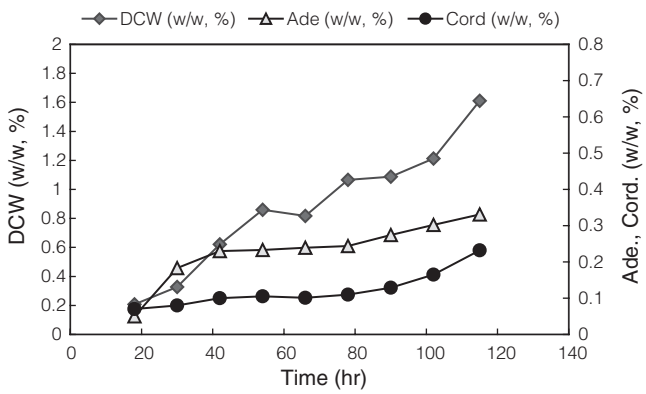

Figure 5. Time course of *A. camphorata* strain BCRC 35396 cultivation in a 100-L fermentor. DCW: dry cell weight (w/w, %); Ade: Adenosine (w/w, %); Cord: cordycepin (w/w, %).

bases in *C. sinensis* and several related fungi. Quantitation of adenosine and profile analysis were carried out by reversed-phase high performance liquid chromatography. The retention time of adenosine and cordycepin was 31 min and 33 min, respectively. Edelson *et al.*[27] used silica-based ion-exchange column for the nucleic acid constituents including adenosine by HPLC method. However, it was not demonstrated clearly how to analyze adenosine and cordycepin simultaneously by gradient elution.

II. *Adenosine and Cordycepin Contents of Cultivated Antrodia camphorata in 5-L Fermentors*

The time course of *A. camphorata* BCRC 35396 strain cultivated in 5-L fermentor is shown in Figure 3. The results revealed that cell growth may reach the stationary phase after 100 hr in 5-L fermentor. The adenosine and cordycepin contents increased significantly within the log phase. Similar results of strain BCRC 35398 are shown in Figure 4. The largest amount of adenosine and cordycepin produced by BCRC 35398 strain were 0.438% and 0.184% (w/w), respectively. There were no significant differences of adenosine and cordycepin contents between the two strains.

III. *Adenosine and Cordycepin Contents of Cultivated Antrodia camphorata in 100-L Fermentors*

The time course of *A. camphorata* BCRC 35396 and BCRC 35398 strain cultivated in 100-L fermentors are shown in Figures 5 and 6. The cell growth might reach the stationary phase after 120 hr of fermentation. The trend of the adenosine and cordycepin contents within the log phase stage had the similarity found in 5-L fermentors. The results showed that the largest amount of adenosine and cordycepin were 0.382% and 0.253% (w/w) respectively in BCRC

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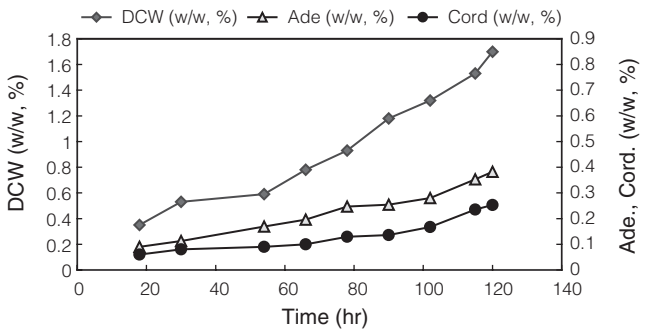

Figure 6. Time course of *A. camphorata* strain BCRC 35398 cultivation in a 100-L fermentor. DCW: dry cell weight (w/w, %); Ade: Adenosine (w/w, %); Cord: cordycepin (w/w, %).

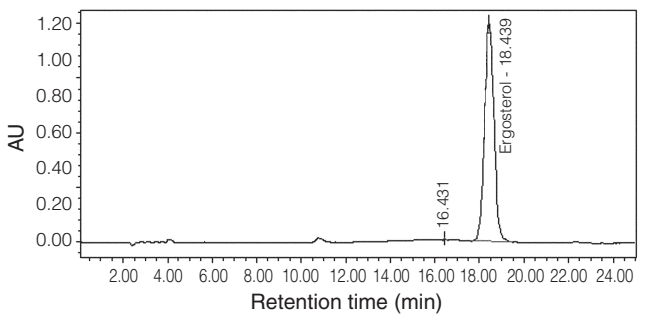

Figure 7. Chromatogram of 0.3 mg/mL standard ergosterol.

35398 strain cultivation. Dry cell mass reached 1.7% (w/w) after 5 days of cultivation.

IV. *Adenosine, Cordycepin and Ergosterol Contents of Cultivated Antrodia camphorata in 700-L fermentors*

The chromatograms of standard ergosterol and sample ergosterol contents of BCRC 35396 strain cultivated in 700-L fermentors are shown in Figures 7 and 8. The adenosine, cordycepin and ergosterol contents of different types of sample cultivated in 700-L fermentor were compared, such as the dried cell after centrifugation and decanted the supernatant of fermentation broth, the spray-dried powder and the whole dried cell without centrifugation. The results are shown in Table 1. It was revealed that adenosine and cordycepin could be found simultaneously in supernatant and whole cells. The contents of adenosine and cordycepin in broth were higher than that in cells. The spray-dried powder possessed less adenosine and cordycepin than that dried in oven, possibly due to temperature effect. The inlet and outlet temperature of the spray-drier reached 192°C and 104°C, respectively, and the oven temperature maintained at 60°C during the drying process. Ergosterol could also be found simultaneously in supernatant and whole cells. The content of ergosterol in broth is less than that in cells. The adenosine, cordycepin and ergosterol contents in the whole dried cell without centrifugation were 5.60 mg/g, 5.00 mg/g and 0.85 mg/g, respectively. The same results were obtained by compared

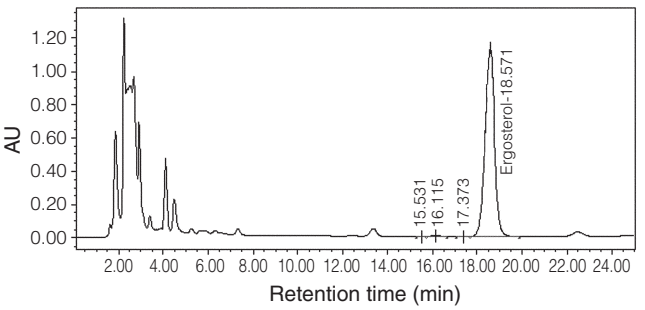

Figure 8. Ergosterol chromatogram of *Antrodia camphorata* sample.

Table 1. Analysis of adenosine, cordycepin and ergosterol contents in different samples of 700-L fermentation broth

Sample	Adenosine (mg/g)	Cordycepin (mg/g)	Ergosterol (mg/g)
Aa	1.90	1.70	8.16
Bb	4.40	4.40	0.98
Cc	5.60	5.00	0.85

[a]The dried cell after centrifugation and decanting the supernatant of fermentation broth.
[b]The spray-dried powder of fermentation broth.
[c]The whole dried cell of fermentation broth without centrifugation.

with different types of sample in 100-L fermentor.

The analysis of adenosine, cordycepin and ergosterol produced by *A. camphorata* mycelia using HPLC and reversed-phase C18 column was developed. The method is rapid, and accurate[23,24,27]. The differences between adenosine and cordycepin contents produced by BCRC 35396 and BCRC 35398 strains cultivated in 5-L and 100-L fermentors were not significant. It was revealed that adenosine and cordycepin could be found simultaneously in supernatant and whole cells. The contents of adenosine and cordycepin in broth were higher than that in cells. On the contrary, the content of ergosterol in broth was less than that in cells. However, comparison of the physiological functions such as antioxidant property, antidote, anti-inflammatory activity and protective effect of the mycelia on chronic liver damage were still ambiguous. Studies on their biological functions are underway.

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