



Effects of additives and bioreactors on cordycepin production from *Cordyceps militaris* in liquid static culture

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Abstract

Cordycepin (3'-deoxyadenosine), a nucleoside analog, was isolated from *Cordyceps militaris*, an entomopathogenic fungus, important in Traditional Chinese Medicine. In this study, Cordycepin production by three strains of *C. militaris* (strains GACP08Y5, GACP08Y1 and GACP0746) in static liquid culture was established using different working volumes and bioreactors. The best cordycepin production of 3005.83 mg/L was obtained by strain GACP08Y5 in 5 L-flasks, containing 2 L medium at day 40, and total cordycepin content reached 6011.66 mg/flask. The utilization ratio of adenine reached 91%. This is the highest report of cordycepin production in a single fermenter. This method provides an effective way for increasing the cordycepin production at a large scale. The strategies used in this study could have a wide application in other fermentation processes.

Key words – comparison – cordycepin – *Cordyceps militaris* – Growth curve – large scale production

Introduction

Cordycepin (3'-deoxyadenosine), a nucleoside analog, was first isolated from *Cordyceps militaris* (Cunningham et al. 1950), and is one of the most important biologically active metabolites of *Cordyceps* species. *C. militaris*, an entomopathogenic fungus belonging to Ascomycota (Sung et al. 2007), has long been used as a Traditional Medicine in China and East Asia (De Silva et al. 2013). Cordycepin has a broad spectrum of biological activity, including anti-cancer (De Silva et al. 2012), anti-tumor (Pao et al. 2012), anti-fungus (Kim et al. 2002), anti-hyperlipidemia (Guo et al. 2010), antioxidant (Ramesh et al. 2012), and anti-leukemia (Thomadaki et al. 2008). Cordycepin is also a Phase I/II clinical stage drug candidate for treatment of refractory Acute Lymphoblastic Leukemia (ALL) patients who express enzyme terminal deoxynucleotidyl transferase (TdT) (ClinicalTrials. Gov, verified by OncoVista, Inc., 2009).

Previous studies have demonstrated that cordycepin could be obtained by chemical synthesis (Aman et al. 2000). However, solid-state fermentation and liquid fermentation are the most popular method to obtain cordycepin. Cordycepin has been extracted from fruiting bodies of *C. militaris* and isolates grown on solid medium (Wen et al. 2014a). In previous studies, it has been shown that formation of fruiting bodies of *C. militaris* needs a long time (60 days) (Wen et al. 2014a, 2014b),

and repeated subculturing results in decreased or no fruiting bodies (Shrestha et al. 2012, Wen et al. 2012). The extraction of cordycepin from fruiting bodies or isolates grown on solid medium is also difficult.

In previous reports, liquid fermentation (submerged culture and surface liquid culture / liquid static culture) was considered to be the effective way for obtain cordycepin. The culture requirements of carbon/nitrogen ratio, dissolved oxygen, salts from deep ocean water, amino acids and nucleoside analogues have been investigated with *C. militaris* by submerged culture. Several of these factors were effective components for improved cordycepin production (Hung et al. 2015, Mao & Zhong 2004, Masuda et al. 2007). Cordycepin has been obtained via surface liquid culture or static liquid culture of strains. The optimum carbon and nitrogen sources, additives and medium depth were studied (Das et al. 2009, Kang et al. 2014, Masuda & Sakurai 2006). In these reports, *C. militaris* mutant was obtained by a new mutagenesis technique called 'ion beam' (Das et al. 2008). The *C. militaris* mutant G81-3 has higher productivity to produce cordycepin in surface culture, and cordycepin production reached 14300 mg/L (Masuda et al. 2014). A highest yield of 1405.94 mg/L was obtained in a single fermenter (1000 mL cylindrical glass bottle containing 700 mL medium) by static liquid culture (Kang et al. 2014).

The aim of this study was to improve cordycepin production by *C. militaris* in static liquid culture, by adding different additives and using different bioreactors. The working volume in different bioreactor was set for *C. militaris* strains (GACP08Y5, GACP08Y1, and GACP0746) and the additives were added to the medium at three different times. Cordycepin production per unit volume and total content of cordycepin in the bioreactor was studied simultaneously.

Materials & Methods

Microorganism and Seed Culture

Three different wild-type strains of *C. militaris*, strains GACP08Y5 and GACP08Y1 (GACP, the Herbarium of Guizhou Agricultural College, Guiyang, Guizhou, China), collected from Yuse National Forest Park in Guizhou Province and strain GACP0746 collected from Mt. Wawu in Sichuan Province, China were used in this study. Strains were isolated from single ascospores (Wen et al. 2014b). Strains were maintained on potato dextrose agar (PDA) slants, following incubation at 25 °C for 7 days and then stored at 4 °C. Sterilized distilled water was added to the slant and the asexual spores washed off and then filtered through sterilized absorbent cotton in infundibulum. The spore suspension of *C. militaris* was added to the seed culture medium at a suitable concentration (Wen et al. 2014a). The seed culture was grown in a 250-mL flask containing 70 mL medium (sucrose 20 g/L; peptone 20 g/L; KH₂PO₄ 1 g/L; and MgSO₄·7H₂O 0.5 g/L; pH natural) at 25 °C on a rotary shaker incubator at 150 r/min for 5 days (Kang et al. 2014).

Static liquid culture, medium and additives

The medium of static liquid culture for *C. militaris* comprised sucrose 20 g/L, peptone 20 g/L and KH₂PO₄ 1 g/L; MgSO₄·7H₂O 0.5 g/L. The pH was natural, followed by autoclaving for 30 min at 121 °C. The static culture experiments were performed in 3 L-flasks, containing 2 L of media (2 L/3 L; v/v), 5 L-flasks containing 2 L, 3 L, and 4 L of media (2 L/5 L, 3 L/5 L and 4 L/5 L; v/v), 370 mL-cylindrical glass bottles (inner diameter 70 mm and height 120 mm) containing 300 mL of media (300 mL/370 mL; v/v) and 480 mL cylindrical glass bottles (inner diameter 80 mm and height 120 mm) containing 400 mL of media (400 mL/480 mL; v/v). The inoculated flasks were sealed with a cotton plug and the bottles sealed by a film of polypropylene plastic. The static liquid culture was started by inoculating the liquid seed with 10% (v/v; the biomass dry weight of seed culture is 54 mg/mL) into bioreactors and incubated at 25 °C for 52 days. Adenine 1 g/L and glycine 16 g/L were added to the medium at 4, 10, and 30 days.

Analytical methods

For the analysis of effects of adenine on cordycepin production, all samples collected at 52

days from different culture bottles were centrifuged at $2810 \times g$ for 20 min. The supernatant was filtered through a $0.45 \mu\text{m}$ membrane and the filtrate was analyzed by high-performance liquid chromatography (1100 series, Agilent Technology, U.S.). Standard adenine and cordycepin (Sigma, USA) were dissolved in distilled water for calibration. The mobile phase was 10 mM KH_2PO_4 , which was dissolved in methanol/distilled water (6:94). Elution was performed at a flow rate of 1.0 mL/min with column temperature at 45°C and UV wavelength of 259 nm (Kang et al. 2014, Wen et al. 2014a). All figures were formed by OriginPro 8.5.0 SR1 b161 software package (OriginLab Corporation, Northampton, USA).

Results

Effects of working volume and additives on cordycepin production for strain GACP08Y5

Effects of 2 L/3 L on cordycepin production

The influence of 2 L/3 L bioreactors on cordycepin production by *C. militaris* was studied in static liquid culture for strain GACP08Y5. As shown in Figure 1b, the production of cordycepin increased gradually with cultivation time from 0 to 52 days. The maximal cordycepin production reached 2402.78 mg/L on day 52, and this value was 7.3 times higher than control (328.57 mg/L on day 52). Production of cordycepin increased rapidly at 2 - 4 days later with the addition of adenine 1 g/L and glycine 16 g/L. In Figure 1a and 1b, the pH value of medium decreased firstly from 0 to 6 days, and then increased slowly from 6 to 52 days. There were three peaks of adenosine concentration were found at 8, 36, and 48 days in experimental group. From Figure 1b, adenine concentration was decreased rapidly at later with adding adenine 1 g/L and glycine 16 g/L. At the end of the fermentation process, residual adenine concentration in the media was 673.03 mg/L, and the utilization ratio of adenine $((3 \text{ g/L} - \text{residual adenine concentration})/3 \text{ g/L} \times 100\%)$ reached 77.57%. The change of adenine concentration shows “A” form in control group (Figure 1a), and the maximal concentration of adenine was reached 11.23 mg/L at day 26.

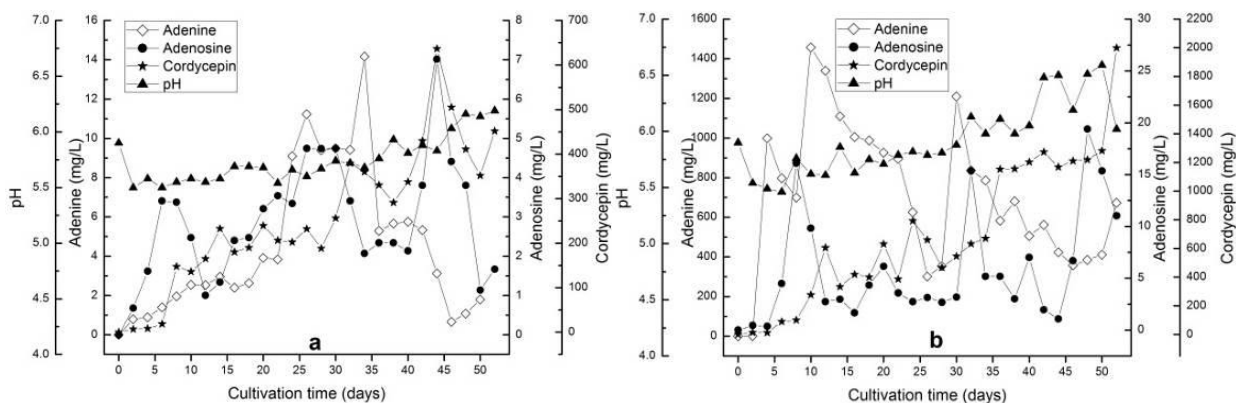


Figure 1 – Effects of 3L-flasks containing 2L medium on cordycepin production for strain GACP08Y5 (a: Control group; b: Experimental group).

Effects of working volume in 5 L-flasks on cordycepin production

In order to evaluate the influence of working volume on cordycepin production, 2 L, 3 L, and 4 L of media were fermented in 5 L-flasks (Figure 2, 3, and 4). Cordycepin production increased gradually from 0 to 40 days, and then decreased slowly from 40 to 52 days (Figure 2b). And in Figure 2b and 3b, the cordycepin production was increased until 52 days. The maximal cordycepin production reached 3005.83 mg/L on day 40, 1391.00 mg/L on day 52, and 895.79 mg/L on day 52 in 2 L, 3 L, and 4 L of medium, respectively. From Figure 2b, and 3b, the pH value of media decreased firstly, and then increased. However, in Figure 4b, the pH value reduced gradually until end of the fermentation process. And we found the ultimate pH values were reduced from 7.36 to 5.27 in three tests. Adenosine concentration was increased rapidly at 2 - 4 days later with adding

adenine 1 g/L and glycine 16 g/L in Figure 2b, 3b, and 4b. Subsequently, they were reduced rapidly. Especially, there was a peak of adenosine concentration at 48 or 50 day, and there was positive correlation between adenosine concentration and working volume. Adenine concentration was reduced rapidly at later with adding adenine 1 g/L and glycine 16 g/L (Figure 2b, 3b, and 4b). Residual adenine concentration in medium were 245.64 mg/L, 888.51 mg/L and 537.26 mg/L at time of the maximal cordycepin production, and the utilization ratio of adenine reached 91.81%, 70.38%, and 82.09% respectively. The change of adenine concentration shows “A” form in control group (Figure 2a, 3a, and 3a), and the highest concentration of adenine were reached 5.00 mg/L, 5.50 mg/L, and 13.10 mg/L at day 24, 30, and 26 respectively.

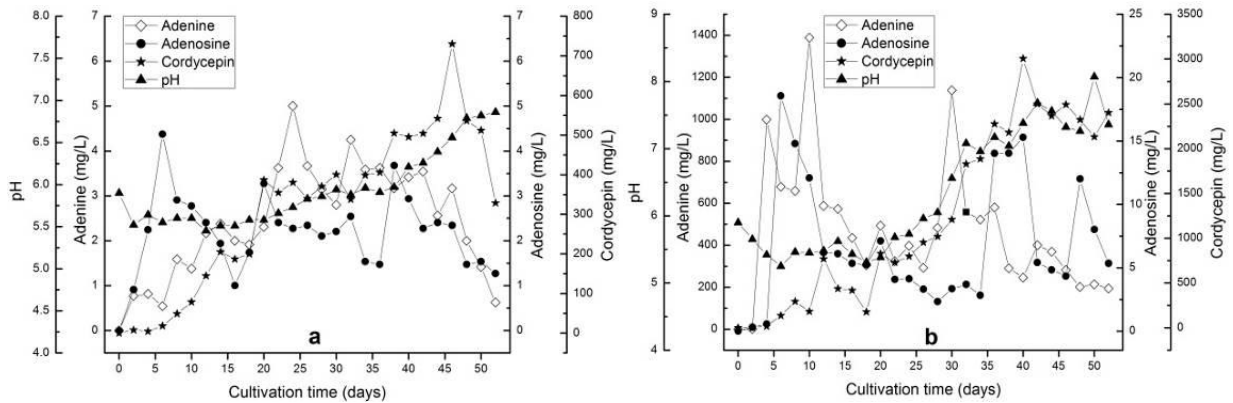


Figure 2 – Effects of 5 L-flasks containing 2 L medium on cordycepin production for strain GACP08Y5 (a: Control group; b: Experimental group).

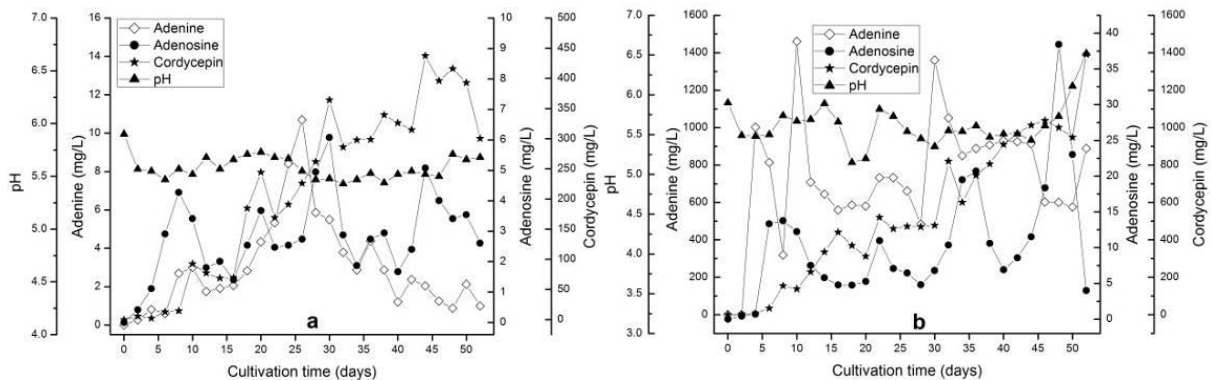


Figure 3 – Effects of 5 L-flasks containing 3 L medium on cordycepin production for strain GACP08Y5 (a: Control group; b: Experimental group).

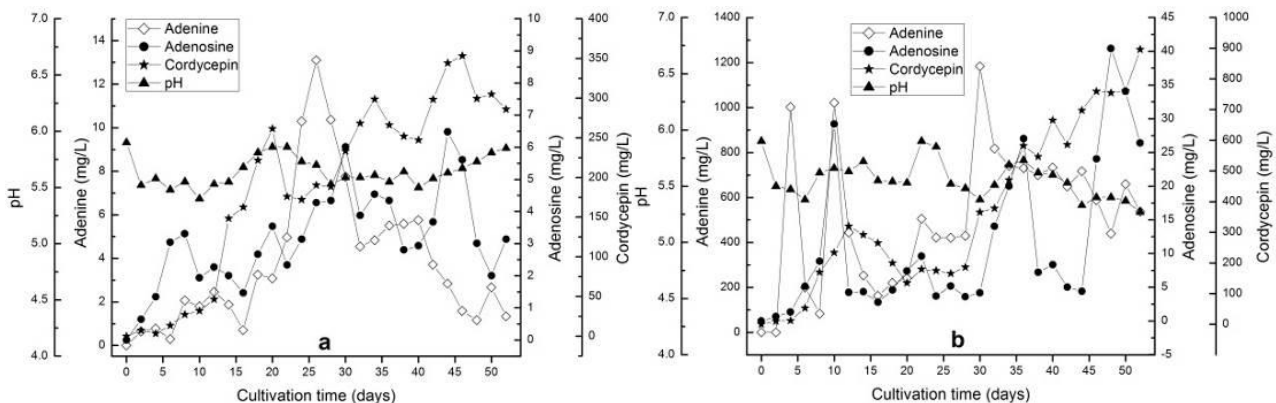


Figure 4 – Effects of 5 L-flasks containing 4 L medium on cordycepin production for strain GACP08Y5 (a: Control group; b: Experimental group).

Effects of 300 mL/370 mL and additives on cordycepin production

In this study, 370 mL-cylindrical glass bottles containing 300 mL medium was studied on cordycepin production for strain GACP08Y5. As shown in Figure 5b, the cordycepin production increased gradually with cultivation time from 0 to 50 days. The result showed that the maximum cordycepin production of 2241.50 mg/L was achieved at day 50, and this value was 3.7 times than control group (605.23 mg/L on day 50). But cordycepin production was reduced slightly at day 52. From Figure 5b, the pH value of media decreased firstly from 5.9 to 4.81, and then increased rapidly from 4.81 to 8.24. In control group, the changing curve of pH value was similar to experimental group (Figure 5a, and 5b). In the same time, we found that the ultimate pH value was higher than initial in experimental group, but the control group was opposite. Adenosine concentration was increased rapidly later with adding adenine 1 g/L and glycine 16 g/L, and then decreased rapidly in Figure 5b. Residual adenine concentration in medium was 802.76 mg/L at time of the maximal cordycepin production, and the utilization ratio of adenine reached 73.24 %. The change of adenine concentration shows “A” form in control group (Figure 5a), and the maximal concentration of adenine was reached 8.10 mg/L at day 24.

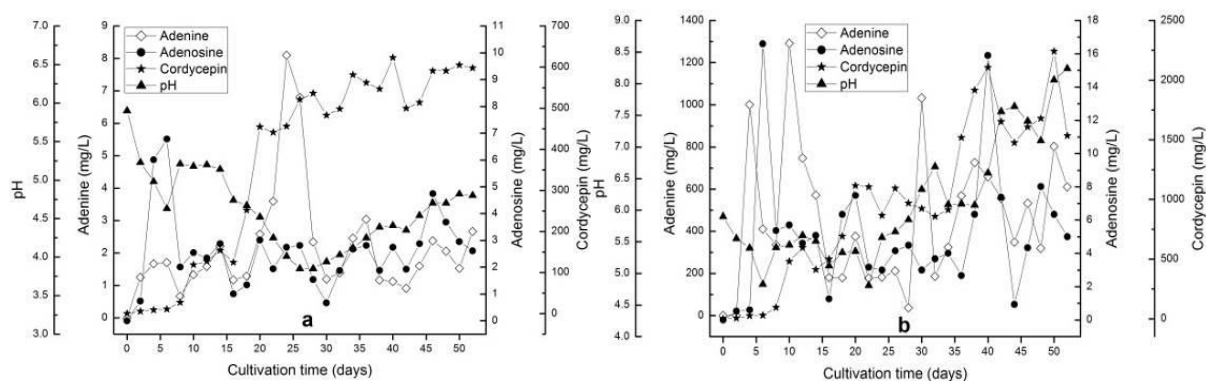


Figure 5 – Effects of 370 mL-cylindrical glass bottles containing 300 mL medium on cordycepin production for strain GACP08Y5 (a: Control group; b: Experimental group).

Effects of 400 mL/480 mL and additives on cordycepin production

In this experiment, 480 mL-cylindrical glass bottles containing 400 mL medium was investigated on cordycepin production for strain GACP08Y5. Our results demonstrated that the cordycepin production was increased gradually with cultivation time from 0 to 52 days. The maximum yield of cordycepin production reached 2440.89 mg/L at day 52, and this value was 4.98 times than control group (491.00 mg/L on day 52) (Figure 6b). The results showed there were differences in cordycepin production of experimental group and control group. The maximum cordycepin production was achieved at day 46 and not day 52 in control group (Figure 6a). The pH value of media decreased firstly, and then increased gradually (Figure 6a and 6b). And the ultimate pH values reached 8.14 at day 52 in experimental group (Figure 6b). Adenosine concentration was increased rapidly later with adding adenine 1 g/L and glycine 16 g/L, and then decreased rapidly in Figure 6b. Residual adenine concentration in medium was 561.53 mg/L at day 52, and the utilization ratio of adenine reached 81.28%. The change of adenine concentration shows “N” form in control group (Figure 6a), and the maximal concentration of adenine was reached 3.30 mg/L at day 52.

Effects of working volume and additives on cordycepin production for strain GACP08Y1 Effects of 2 L/3 L and additives on cordycepin production

To examine the effect of working volume and additives on the production of cordycepin, 3 L-flask containing 2 L medium were used for cultivation of *C. militaris* GACP08Y1 strains. As shown in Figure 7b, the cordycepin production increased gradually with cultivation time from 0 to 42 days, and then decreased slowly from 42 to 52 days. The maximum cordycepin production of

2705.8 mg/L was achieved, and this value was 1.77 times than control group (1530.15 mg/L on day 42). However, the maximum cordycepin production was achieved at day 48 and not day 52 in control group (Figure 7a). In experimental group, the pH value of media was decreased firstly, and then increased gradually. Nevertheless, it was decreased gradually until end of the fermentation process in control group. The adenine concentration was decreased rapidly later with adding additives (Figure 7b). Residual adenine concentration in medium was 446.71 mg/L at day 52, and the utilization ratio of adenine reached 85.11%. The change of adenine concentration shows “A” form in control group (Figure 7a), and the maximal concentration of adenine was reached 14.46 mg/L at day 18.

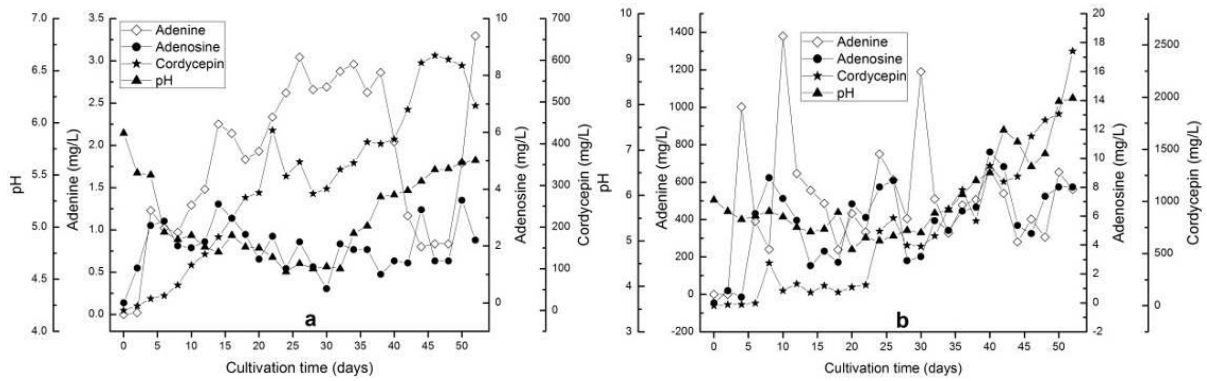


Figure 6 – Effects of 480 mL-cylindrical glass bottles containing 400 mL medium on cordycepin production for strain GACP08Y5 (a: Control group; b: Experimental group).

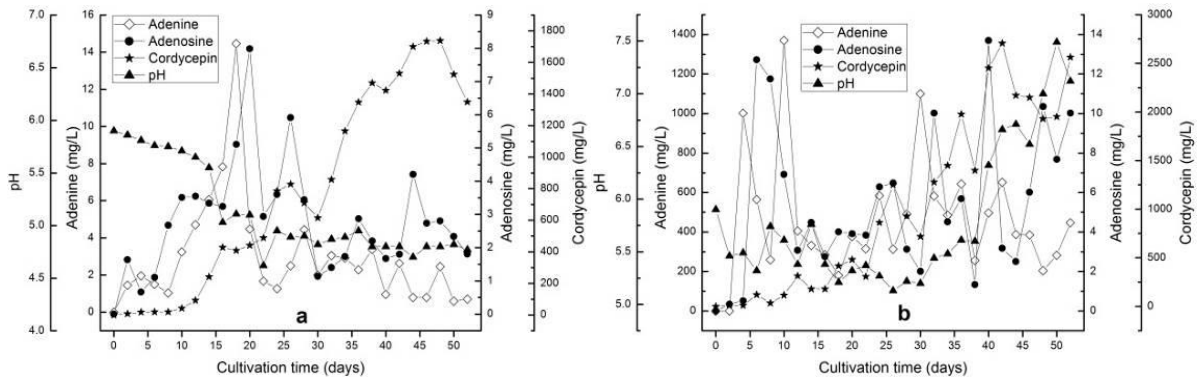


Figure 7 – Effects of 3 L-flasks containing 2 L medium on cordycepin production for strain GACP08Y1 (a: Control group; b: Experimental group).

Effects of 300 mL/370 mL and additives on cordycepin production

To investigate the influence of working volume and additives on the production of cordycepin, the fungus of *C. militaris* GACP08Y1 strains was cultivated in medium of 370 mL-cylindrical glass bottles containing 300 mL medium. As shown in Figure 8b, the cordycepin production increased gradually until day 52. The highest cordycepin production of 4008.32 mg/L was obtained, and this value was 3.96 times than control group (1011.29 mg/L) on day 52. However, the highest cordycepin production 1304.02 mg/L was achieved at day 44 and not day 52 in control group (Figure 8a). From Figure 8b, the pH value of media was decreased firstly, and then increased rapidly. Nevertheless, it was decreased gradually until end of the fermentation process in control group. The adenine concentration was decreased rapidly later with adding adenine 1 g/L and glycine 16 g/L (Figure 8b). Residual adenine concentration in medium was 402.00 mg/L at day 52, and the utilization ratio of adenine reached 86.6%. The change of adenine concentration shows “M” form in control group (Figure 8a), and two peaks of adenine concentration were found at 14 and 36 days.

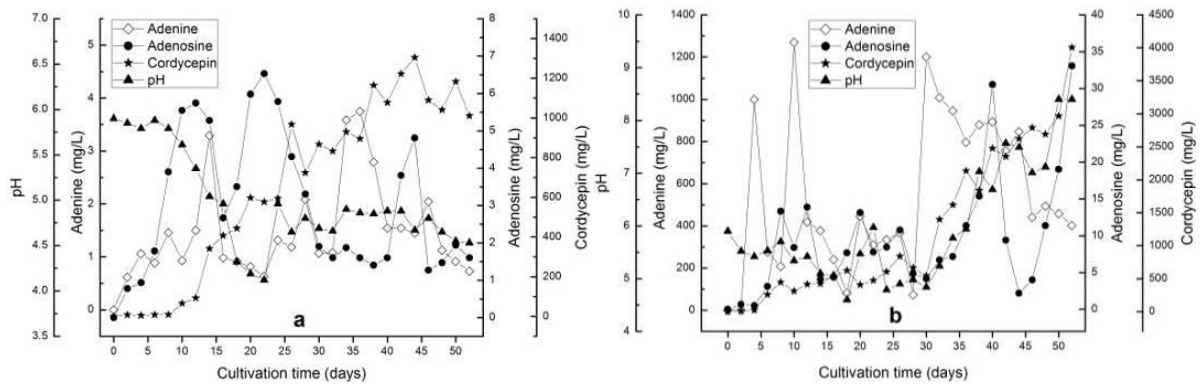


Figure 8 – Effects of 370 mL-cylindrical glass bottles containing 300 mL medium on cordycepin production for strain GACP08Y1 (a: Control group; b: Experimental group).

Effects of 400 mL/480 mL and additives on cordycepin production

In this study, 480 mL-cylindrical glass bottles containing 400 mL medium was examined on cordycepin production for strain GACP08Y1. As shown in Figure 9b, the cordycepin production increased rapidly with cultivation time from 0 to 40 days, and then decreased slowly from 40 to 52 days. The maximum cordycepin production of 4376.96 mg/L was achieved at day 46. In control group, the tendency of cordycepin production was similar to experimental group. However, the maximum cordycepin production was obtained at day 44, and then decreased rapidly. From Figure 9b, the pH value of media decreased firstly from 5.9 to 4.16, and then increased rapidly from 4.16 to 8.02. In control group, the changing curve of pH value was similar to experimental group (Figure 9a, and 9b). In the same time, we found that the ultimate pH value was higher than initial in experimental group, but the control group was opposite. Adenosine concentration was increased rapidly later with adding adenine 1 g/L and glycine 16 g/L, and then decreased rapidly in Figure 9b. Residual adenine concentration in medium was 177.24 mg/L at time of the maximal cordycepin production, and the utilization ratio of adenine reached 94.09%. The change of adenine concentration was fluctuation (Figure 9a), and the maximal concentration of adenine was reached 4.04 mg/L at day 48.

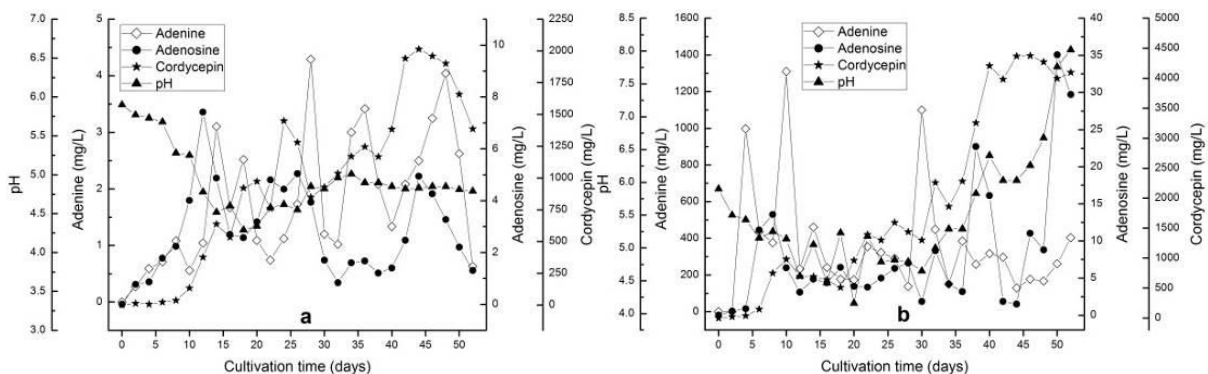


Figure 9 – Effects of 480 mL-cylindrical glass bottles containing 400 mL medium on cordycepin production for strain GACP08Y1 (a: Control group; b: Experimental group).

Effects of working volume and additives on cordycepin production for strain GACP0746
Effects of 2 L/3 L and additives on cordycepin production

In this experiment, 3 L-flasks containing 2 L medium was investigated on cordycepin production for strain GACP0746. The results demonstrated that the cordycepin production increased gradually with cultivation time from 0 to 40 days, and then decreased from 40 to 46 days, and increased gradually at the end of the fermentation process (from 46 to 52 days). The highest yield of cordycepin production reached 2143.11 mg/L at day 52 (Figure 10b). The changing curve

of cordycepin production shows “M” form in control, and two peaks of cordycepin production were found at 24 and 45 days, respectively. The highest yield of cordycepin production was 460.51 mg/L at day 45 (Figure 10a). There were similar change about the pH value of media in experimental group and control group (Figure 10a and 10b). They were decreased firstly, and then increased slowly respectively. Adenosine concentration was increased rapidly later with adding adenine 1 g/L and glycine 16 g/L, and then decreased rapidly in Figure 10b. Adenine was added to the medium as additive at 4, 10, and 30 days. As shown in Figure 10b, concentration of adenine was decreased rapidly at later with adding adenine 1 g/L and glycine 16 g/L. The residual adenine concentration in medium was 170 mg/L at day 52, and the utilization ratio of adenine reached 94.33%. The change of adenine concentration was fluctuation (Figure 10a), and approximate “A” form. The maximal concentration of adenine was reached 2.96 mg/L at day 46.

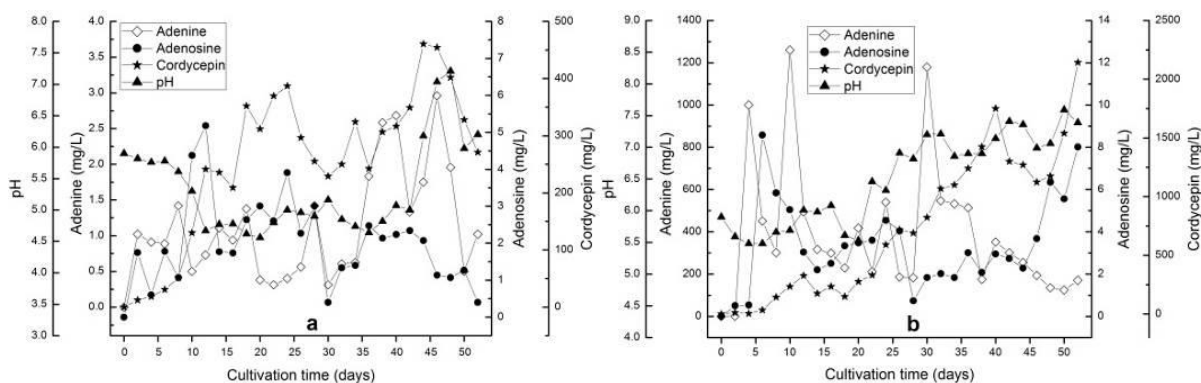


Figure 10 – Effects of 3L-flasks containing 2L medium on cordycepin production for strain GACP0746 (a: Control group; b: Experimental group).

Effects of 300 mL/370 mL and additives on cordycepin production

In this test, 370 mL-cylindrical glass bottles containing 300 mL medium was investigated for cordycepin production using strain GACP0746. As shown in Figure 11b, the cordycepin production increased gradually with cultivation from 0 to 50 days. The highest yield of cordycepin reached 2346.09 mg/L. There were differences between the experimental group and control group. The peak of cordycepin production was at 44 days, and then decreased in the control group (Figure 11a). The pH value of media first decreased, and then increased in both the experimental group and control group (Figure 11a and 11b). The optimal pH value was higher than initial in experimental group, but the control group was opposite. Adenosine concentration was increased rapidly later with adding adenine 1 g/L and glycine 16 g/L, and shows “N” form in experimental group (Figure 11b). From Figure 11b, concentration of adenine was decreased rapidly at later with adding adenine 1 g/L and glycine 16 g/L. The residual adenine concentration in medium was 369.49 mg/L at day 50, and the utilization ratio of adenine reached 87.68%. The change of adenine concentration shows “M” form in control group (Figure 11a), and two peaks of adenine concentration were found at 14 and 21 days.

Effects of 400 mL/480 mL and additives on cordycepin production

To investigate the effect of working volume on the production of cordycepin, *C. militaris* strain GACP0746 was cultivated in 480 mL-cylindrical glass bottles containing 400 mL media. Cordycepin production increased gradually until day 52 (Figure 12b). The highest cordycepin production of 2828.12 mg/L was obtained on day 52, and this value was 3.96 times than control group (505.69 mg/L). However, the highest cordycepin production of 790.09 mg/L was achieved at day 46 and not day 52 in the control group (Figure 12a). From Figure 12b, the pH value of media was first decreased, and then increased rapidly. However, the pH decreased gradually until end of the fermentation process in the control group. Adenosine concentration was increased rapidly later with adding additives. The adenine concentration was decreased rapidly later with adding adenine 1

g/L and glycine 16 g/L (Figure 12b). Residual adenine concentration in medium was 558.2 mg/L at day 52, and the utilization ratio of adenine reached 81.39%. The change of adenine concentration shows “M” form in control group (Figure 12a), and two peaks of adenine concentration were found at 14 and 32 days.

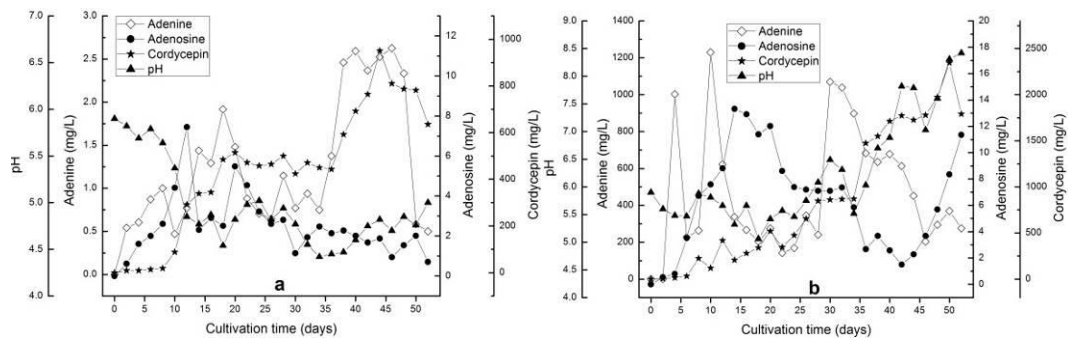


Figure 11 – Effects of 370 mL-cylindrical glass bottles containing 300 mL medium on cordycepin production for strain GACP0746 (a: Control group; b: Experimental group).

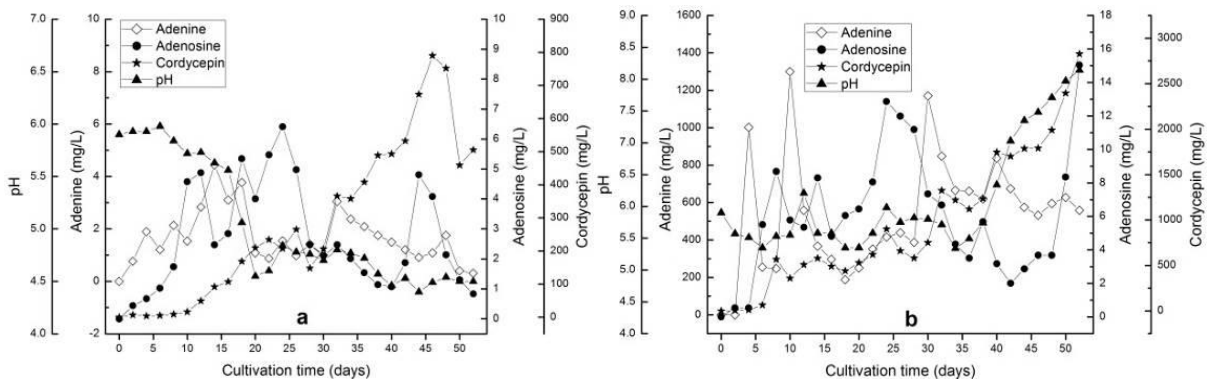


Figure 12 – Effects of 480 mL-cylindrical glass bottles containing 400 mL medium on cordycepin production for strain GACP0746 (a: Control group; b: Experimental group).

Cordycepin production and adenine concentrations in different bioreactors

We compared the differences in cordycepin production, total cordycepin content, adenine concentration, adenine utilization ratio, and cultivation time by adding additives in the different static liquid bioreactors (Table 1). The maximum cordycepin production of 3005.83 mg/L was obtained for *C. militaris* strain GACP08Y5 when adding adenine 1 g/L and glycine 16 g/L in 5 L-flasks containing 2 L medium at day 40. The total content of cordycepin reached 6011.66 mg, and utilization ratio of adenine reached 91.81%. Cordycepin production by *C. militaris* strain GACP08Y1, reached a maximum of 4376.96 mg/L in 480 mL-cylindrical glass bottles containing 400 mL medium at day 46, and utilization ratio of adenine reached 94.09% and was the highest cordycepin production in this study. The maximum total content of cordycepin reached 5127.44 mg in 3 L-flask containing 2 L medium at day 40. The maximum cordycepin production by *C. militaris* strain GACP0746 reached 2828.12 mg/L in 480 mL-cylindrical glass bottles containing 400 mL medium at day 52. However, the maximum total content of cordycepin reached 4286.22 mg in 5 L-flasks containing 2 L medium at day 52. 480 mL-cylindrical glass bottles containing 400 mL medium increased cordycepin production per unit volume for three strains (GACP08Y5, GACP08Y1 and GACP0746).

Nevertheless, 2 L of media in 5 L-flask was an effective working volume for total content of cordycepin. In conclusion, 5 L-flasks containing 2 L with adenine 1 g/L and glycine 16 g/L by *C. militaris* strain GACP08Y5 was the optimal culture method in static liquid culture. Furthermore, 5 L-flasks containing 2 L for *C. militaris* GACP08Y1 was also considered auxiliary cultural method.

Table 1. Cordycepin production in different bioreactors ^a

Strains	Working volume of the medium (v/v) ^b	Cultivation time (d) ^c	Cordycepin production (mg/L)		Total content of cordycepin (mg) ^e		Residual adenine concentration (mg/L)	Utilization ratio of adenine (%) ^f
			Control	Experimental ^d	Control	Experimental		
GACP08Y5	2/3	52	328.57	2402.78	657.14	4805.56	673.03	77.57
	2/5	40	495.26	3005.83	990.52	6011.66	245.64	91.81
	3/5	52	328.57	1391.00	985.71	4173.00	888.51	70.38
	4/5	52	286.12	895.79	1144.48	3583.16	537.26	82.09
	300/370	50	605.23	2241.50	181.57	672.45	802.76	73.24
	400/480	52	491.00	2440.89	235.68	1171.63	561.53	81.28
GACP08Y1	2/3	52	1349.42	2563.72	2698.84	5127.44	446.71	85.11
	300/370	52	1011.29	4008.32	303.39	1202.50	402.00	86.60
	400/480	46	1959.00	4376.96	940.32	2100.94	177.24	94.09
GACP0746	2/3	52	271.00	2143.11	542.00	4286.22	170.00	94.33
	300/370	50	781.698	2346.09	234.51	703.83	369.49	87.68
	400/480	52	505.69	2828.12	242.73	1357.50	558.20	81.39

^a Effects of working volume on cordycepin production, adenine and residual adenine concentrations in different bioreactors with the supplement of additives for *C. militaris* strains.

^b Working volume was the volume of medium in different bioreactors, and was showed by the volume of medium / the volume of bioreactor (v/v).

^c Cultivation time was the time of the maximal cordycepin production.

^d The additives (adenine 1 g/L; glycine 16 g/L) were added to the medium at day 4, 10, and 30.

^e Total content of cordycepin = cordycepin production (mg/L) × working volume (mL).

^f Utilization ratio of adenine = (3 g/L-Residual adenine concentration)/3 g/L×100%.

Discussion

In this paper, different bioreactors, working volumes, and different strains of GACP08Y5, GACP08Y1 and GACP0746 were quantified for their abilities to produce cordycepin in static liquid culture. Cordycepin production, total content of cordycepin, adenine concentration, adenine utilization ratio, and cultivation time differed under the various conditions. The result shown that the cordycepin production increased gradually with cultivation time in control group and experimental group (Figure 1 to 12), and there were significant differences between and experimental set-ups. 5L-flasks containing 2 L and 480 mL-cylindrical glass bottles containing 400 mL of media enhanced cordycepin production per unit volume for strain GACP08Y5, and strains GACP08Y1 and GACP0746 (Table 1). Maximum total cordycepin production per unit volume reached 4376.96 mg/L for *C. militaris* strain GACP08Y1 in 480 mL-cylindrical glass bottles containing 400 mL medium at day 46. This is lower than previous reports 8570 mg/L (Das et al. 2009) and 14300 mg/L (Masuda et al. 2014). However, the total content of cordycepin (3939.26 mg) was higher than previous reports (857 mg (Das et al. 2009) and 2145 mg (Masuda et al. 2014)). A higher value of 6011.66 mg for *C. militaris* GACP08Y5 was achieved in 5 L-flask containing 2 L medium at day 40 (3005.83 mg/L in per unit volume). This is the highest report of cordycepin production in a single fermenter.

In this study, adenine 1 g/L, glycine 16 g/L were added to the medium at day 4, 10, and 30 and clearly enhanced cordycepin production. These results were higher than previously reported (Das et al. 2009, Kang et al. 2014, Masuda et al. 2014, Masuda et al. 2007). This is an excellent method for improved production of cordycepin. Adenine concentration first increased, and later decreased in control group (Figure 1a to 12a). The adenine concentration decreased rapidly when adding adenine 1 g/L and glycine 16 g/L (Figure 1b to 12b). Therefore, we suspect that adenine was first produced, and then cordycepin was biosynthesized with adenine by *C. militaris* in the control group. However, cordycepin was directly synthesized with adenine of additives by *C. militaris* in experimental group. The results show that exogenously supplied adenosine was effective significantly to cordycepin biosynthesis (Chassy & Suhadolnik 1969). Previous research showed that glycine was good additive for increase cordycepin production (Das et al. 2009, Kang et al. 2012, Masuda et al. 2007). The glycine only as additive was added to medium in this study. We

did not detect the change of glycine concentration.

In order to obtain higher total cordycepin content, we used different working volumes of media in different bioreactors. Previous studies have shown that dissolved oxygen (DO) concentration is a key factor in the media for cell growth and metabolite biosynthesis (Mao & Zhong 2004), and was not only an important part of the respiratory chain, but also of metabolite composition (Xie et al. 2008). But there exist limit value of DO in the medium (Mao & Zhong 2004). As shown in Figure 2 to 4 and Table 1, the production of cordycepin reduced gradually with increasing working volume of the medium from 2 to 4L in 5L-flasks, and the total content of cordycepin increased slowly. The DO concentration in the media is considered under the limit value of DO (data not shown) (Kang et al. 2014, Masuda & Sakurai 2006). The results suggest that the limit value of bioreactor and working volume are pivotal in large-scale production by static liquid culture.

In the present experiment, three different wild-type strains of *C. militaris* were studied for production of cordycepin. The ability to produce cordycepin differed between these strains (Table 1). Maximum cordycepin production of 4376.96mg/L was obtained. However, it was obviously lower than previous reports (Das et al. 2009, Masuda et al. 2014). In previous report, high-energy ion beam irradiation was applied to obtain a mutant strain of *C. militaris* with a higher cordycepin production (Das et al. 2009), and the maximum cordycepin production was reached 14300 mg/L (Masuda et al. 2014). Therefore, Screening of the microbial strains was very important for the production of the metabolites.

Simultaneously, mechanism of cordycepin biosynthesis is the most important. Nevertheless, Up to now related gene expression of cordycepin biosynthesis and change of metabolic are still not explained reasonably. Lennon & Suhadolnik (1976) consider that the formation of 3'-deoxyadenosine (cordycepin) may proceed by a reductive mechanism similar to that for the formation of 2'-deoxynucleotides. The pur cluster which encodes the puromycin biosynthetic pathway was studied in *Streptomyces alboniger*, and the oxidation-reduction of 3'-OH for puromycin has been deduced according to similarities of results and to previous biochemical work by authors (Tercero et al. 1996). The result might provide much convincing evidence for the cordycepin biosynthetic pathway (oxidation-reduction of 3'-OH).

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