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Efficient Production of Anticancer Agent Cordycepin by Repeated Batch Culture of *Cordyceps militaris* Mutant

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Abstract*- Cordycepin (3′-deoxyadenosine) exhibits antimicrobial, anticancer, antimetastatic, immunomodulatory and insecticidal effects, and is one of the most important bioactive anticancer agents found in *Cordyceps* species (summer grass-winter worm). However, due to the requirements of specific hosts and strict growth environments, *Cordyceps militaris* is very scarce in nature. Therefore, the production of the anticancer agent cordycepin from *Cordyceps militaris* in a large scale is currently an acute issue that might be solved by a repeated batch operation of surface liquid culture. In a course of searching a suitable culture technique, the repeated batch culture of control and, mutant with an optimized medium for each were used. The medium compositions were 62.6 g/l glucose, 72.5 g/l Yeast Extract (YE); and 86.2 glucose, 93.8 g/l YE for the control, and mutant, respectively. The cordycepin productivities [g/(l·d)] of batch and repeated batch were 0.16, 0.18, and 0.24, 0.44 for control, and mutant, respectively. Moreover, the productivity of the mutant in batch and repeated batch cultures were respectively increased to 0.26 g/(l·d) and 0.48 g/(l·d) by the addition of adenosine to the optimized medium. This is the highest cordycepin productivity [0.48 g/(l·d)] reported until now. In fact, the cordycepin productivities of the repeated batches were 34%, 86% and 82% higher than those of batches for control, mutant, and mutant with adenosine, respectively; whereas, those of mutant and mutant with adenosine were 113% and 131% higher than that of the control, respectively, indicating effectiveness of the repeated batch culture. The nucleic acid-related compounds analysis revealed that the major portion of cordycepin existed in the medium. Therefore, the *Cordyceps militaris* mutant (G81-3) obtained by a proton beam irradiation with the best additive concentration (adenosine 6 g/l) might be an effective combination for repeated batch culture in the optimized medium to achieve a further higher cordycepin productivity.

Keywords-efficient cordycepin production; Cordyceps militaris mutant; repeated batch culture; surface liquid culture

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I. INTRODUCTION

It has been a difficult path to find a suitable method to increase the production of the anticancer agent cordycepin (3′-deoxyadenosine) from the medicinal fungus *Cordyceps militaris*. This novel biometabolite cordycepin has a number of valuable applications, not only against cancer, but also against some other diseases as reported by biotechnologists and medical researchers [1], [2]. In our previous experiments, a new mutant of the *Cordyceps militaris* was obtained using ion beam irradiation technology [3]. In the surface liquid culture of this mutant, it was also evident that the biosynthesis of cordycepin can be regulated by the concentrations of the components in the culture medium, especially the concentrations of the carbon and nitrogen sources, and some additives that have been investigated in our previous studies [4]. The medium components for the control and mutant were respectively optimized as 62.6 g/l glucose, 72.5 g/l YE and 86.2 glucose, 93.8 g/l YE with a cordycepin production of 2.45 g/l and 6.84 g/l [5]. Thereafter, a higher cordycepin production (8.57 g/l) was attained using 6 g/l adenosine as an additive in the optimized medium for the mutant [6].

It is well known that the greatest gain in productivity can be achieved by a vicious culture system rather than one cycle cell culture technique, as the fungal cells grow substantially slower in such a one cycle culture system due to lack of required nutrition supply at the end of the each culture cycle. Whereas, in a repeated batch culture technique, the unuseful medium is removed at the end of each batch and is replenished with a fresh medium having a same medium composition. The addition of a fresh medium and elimination of an unuseful one at the end of each cycle/batch provides the cells with the environment they require actually to achieve a higher productivity. Therefore, the conventional culture system is not prospective as the mycelial mat can be used only one time and usually accompanied by a lower productivity, on the other hand, in the case of repeated batch culture, the mycelial mat can be used usually 3-5 times and may provide a higher productivity. Therefore, a repeated batch culture was investigated in the present study to achieve a higher cordycepin production.

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II. MATERIALS AND METHODS

2.1 Fungal strain, media and stock culture

Cordyceps militaris NBRC 9787 used in the present experiments as the control (wild strain), and a prospective mutant of the said strain obtained by ion beam irradiation (G81-3) were stored on a PDA (Nissui Pharmaceutical Co., Ltd., Japan) slant at 5 °C.

2.2 Surface liquid culture using control and mutant (G81-3) of C. militaris

The surface liquid culture methodology is as described in our previous study [3], but the culture was started by inoculating two seed disks (instead of single disk per bottle) into a 500 ml culture bottle. All experiments were carried out at least in duplicate, and the results were averaged. The compositions of the optimized media for the control and mutant used in this experiment are shown in Table I. For the mutant, the optimized medium with the best additive concentration (adenosine 6 g/l) attained in the preceding experiment were also used.

2.3 Repeated batch culture

A medium exchanging port was placed at the same position as the sampling port in the 500 ml surface liquid culture bottle. In addition, the supporter, which was made of stainless steel mesh (wire diameter, φ=0.3mm) was placed in the bottle in order to prevent the biofilm from soaking in the liquid medium. The culture broth replaced with a fresh medium through the medium exchanging port using a syringe as soon as the glucose concentration tends to zero (glucose conc. <0.5% of the initial conc.) (Figs. 1a, and 1b). Three repeated batch cultures were conducted simultaneously using the control with medium A, mutant with medium B, and mutant with medium C (medium B plus adenosine 6 g/l).

2.4 Preparation of extract from the mycelia of C. militaris

The cultured mycelia of *C. militaris* were separated from the medium and sufficiently rinsed with distilled water. The mycelia were dried in a vacuum drying oven at 40°C for 24 h and then ground into a powder. The dry powder (0.5 g) was suspended in 10ml of distilled water and sonicated for 30 min at 20KHz, and 100 W (XL2020, Heat System Inc., USA) in an ice bath. The supernatant obtained by centrifugation of this homogenate was used as the extract solution from the mycelia.

2.5 Analytical procedures

The cordycepin concentration was determined by an HPLC (LC-9A system, Shimadzu Corp., Japan) under the following conditions: column, TSK-gel ODS-80Ts (Tosoh Corp., Japan); mobile phase, methanol and 0.1% phosphoric acid (2/98, v/v); flow rate, 1.0 ml/min; column temperature, 40 °C and peak detection, UV at 260 nm. The cordycepin concentration shown in this experiment was re-estimated by considering the condensation of the medium in the culture bottle due to vaporization.

The glucose concentration was analyzed by the mutarotase-glucose oxidase method using the Glucose CII test Wako (Wako Pure Chemical Industries, Ltd., Japan). The pH of the collected samples was measured by a pH meter.

The concentrations of the nucleic acid-related compounds other than cordycepin in the filtrate and the extract were also determined by the same HPLC system as the cordycepin measurement under different elution condition. The mobile phase was prepared from the two solvent systems (A, 2.0% MeOH in 0.01 M $(NH_4)H_2PO_4$; B, 20% MeOH in 0.01 M $(NH_4)H_2PO_4$). After solution "A" was used as the mobile phase for 20 min, the linear gradient was performed by decreasing the ratio of "A" from 100% to 0% for 10 min. For the next 10 min, 100% of "B" was used as the mobile phase. The overall elution time was 40 min. The flow rate was 0.8 ml/min, and the column temperature was 40 °C. The authentic standards were purchased from the Wako Pure Chemical Industries, Ltd., Japan.

III. RESULTS AND DISCUSSION

3.1 Cordycepin production by batch and repeated batch cultures

3.1.1 Typical batch culture of C. militaris

Three batch cultures were performed using control and mutant with the medium optimized for control (medium A) and mutant (medium B). In addition, adenosine supplemented medium was used for the mutant (medium C). The surface of the liquid medium was covered with biofilm by 15 days for the mutant and 12 days for the control. Figs. 2-4 show the typical time courses of cordycepin production, glucose, and pH by surface liquid culture using the control (medium A) and, mutant (medium B and C).

The cordycepin production started slightly after the starting of the cell growth and almost stopped when the glucose consumption was finished. When the residual glucose reached 0, there was a rise in pH due to autolysis leading to the maximum cordycepin production. The production and productivities of cordycepin were 2.33, 0.16; 5.68, 0.22 and 7.34 g/l, 0.26 g/(l·d) for control, mutant and mutant with adenosine in batch cultures, respectively (Figs. 2-4; Table II). It can be mentionable that the present cordycepin productions were somewhat lower than those obtained in our previous experiment [5]. In fact, cordycepin production was calculated in this experiment on the day having the highest productivity in each case in contrast to that of the control (Table II), which is practically a few days before reaching the production peak.

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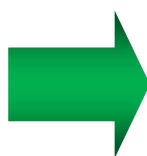
TABLE I. COMPOSITION OF OPTIMIZED MEDIA FOR REPEATED BATCH CULTURE (MUTANT AND CONTROL)

Components	Concentration (g/l)		
	A. Control	B. Mutant	C. Mutant +Adenosine
Nitrogen source			
Yeast extract	72.5	93.8	93.8
Carbon source			
Glucose	62.6	86.2	86.2
Additive			
Adenosine			6
Others (diluted to 1/10 concentration of Vogel's medium) (Same for both mutant and control)			
$NaOC(COOH)(CH_2COONa)_2 \cdot 2H_2O$		0.28	
KH_2PO_4		0.5	
NH_4NO_3		0.2	
$MgSO_4 \cdot 7H_2O$		0.02	
$CaCl_2 \cdot 2H_2O$		0.01	
Citric acid		0.46×10^{-3}	
$ZnSO_4 \cdot 7H_2O$		0.50×10^{-3}	
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$		0.10×10^{-3}	
$CuSO_4 \cdot 5H_2O$		0.025×10^{-3}	
H_3BO_3		5.0×10^{-6}	
$MnSO_4 \cdot (4–5)H_2O$		5.0×10^{-6}	
$Na_2MoO_4 \cdot 2H_2O$		5.0×10^{-6}	



(a) Old medium drawn

(b) With a fresh medium

Figure 1 (a, b). Replenishment of culture medium in a repeated batch culture

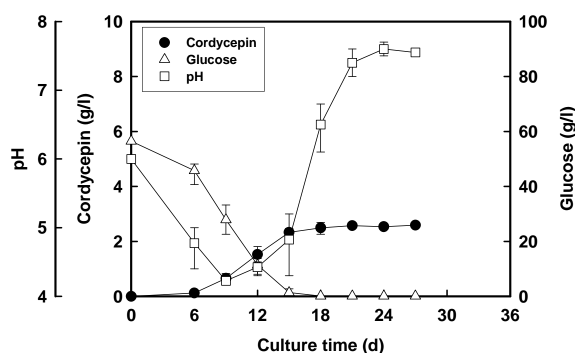


Figure 2. Typical time course of the surface liquid culture using control

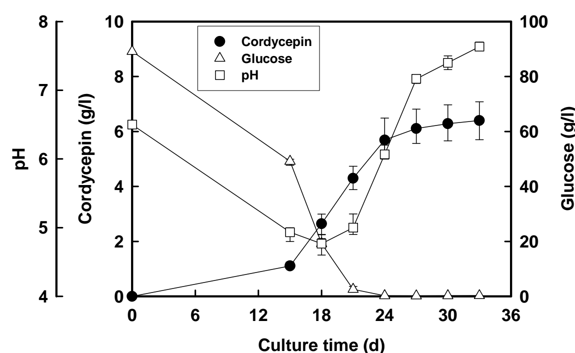


Figure 3. Typical time course of the surface liquid culture using mutant G81-3

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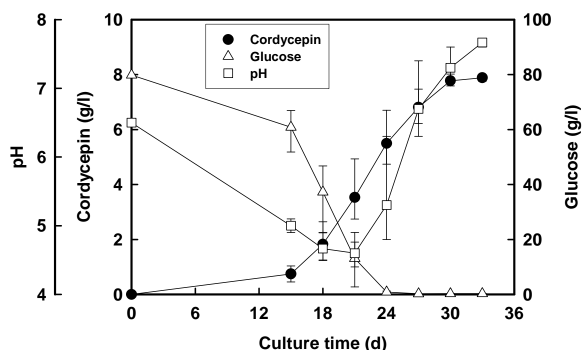

Figure 4. Typical time course of the surface liquid culture using mutant G81-3 with adenosine

3.1.2 Repeated batch culture of C. militaris

For a further increase in cordycepin production, the feasibility of the repeated batch cultures was examined. In our present culture technique, steel mesh was used to replenish the medium without causing any harm to the surface biofilm. The glucose consumption was used as a criterion for the exchange of medium. Figs. 5 (a), 6 (a) and 7 (a) show the time courses of the cordycepin production and Figs. 5 (b), 6 (b) and 7 (b) show the glucose concentrations and pH of the control with medium A and, mutant with media B and C, respectively.

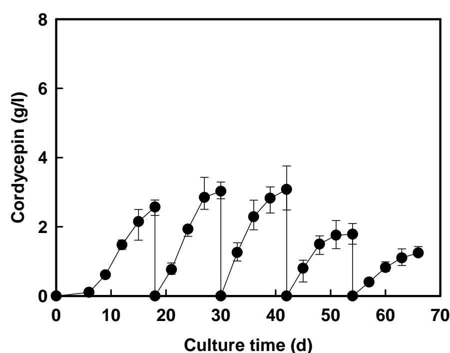

Figure 5 (a). Time courses of the cordycepin production of repeated batch culture using control

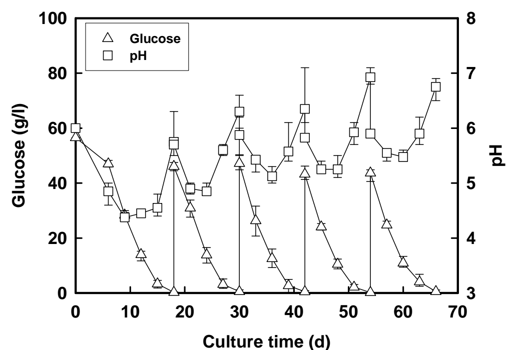

Figure 5 (b). Time courses of the glucose concentration and pH of repeated batch culture using control

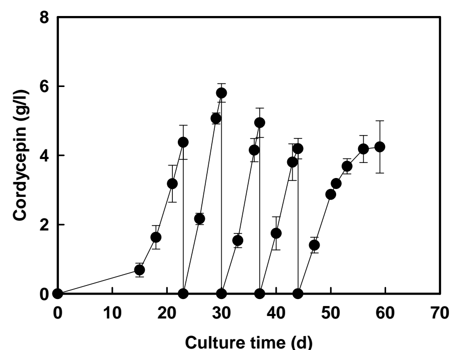

Figure 6 (a). Time courses of the cordycepin production of repeated batch culture using mutant

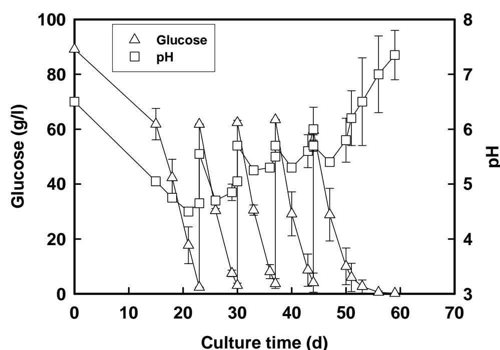

Figure 6 (b). Time courses of the glucose concentration and pH of repeated batch culture using mutant

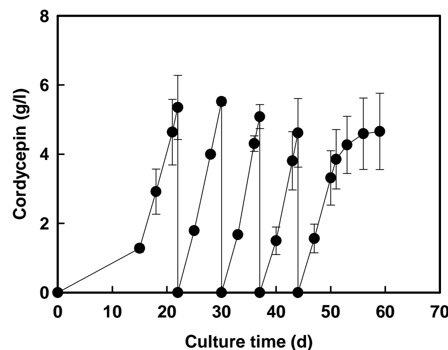

Figure 7 (a). Time courses of the cordycepin production of repeated batch culture using mutant with adenosine

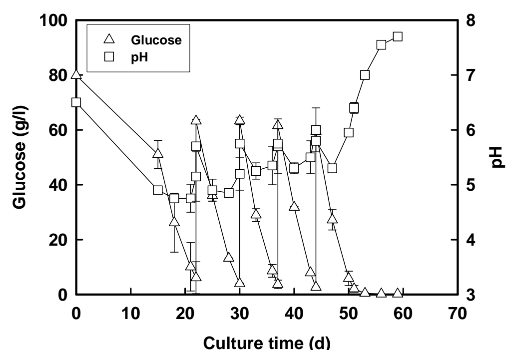

Figure 7 (b). Time courses of the glucose concentration and pH of repeated batch culture using mutant with adenosine

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TABLE II. COMPARISON OF CORDYCEPIN PRODUCTIVITY IN BATCH AND REPEATED BATCH CULTURES

	Control	Mutant	
Culture types	A. Optimized medium	B. Optimized medium	C. Optimized medium + Adenosine
Batch			
Period (d)	15	24	24-30
Production (g/l)	2.33	5.68	7.34
Productivity [g/(l·d)]	0.155	0.237	0.263
Repeated batch			
Period (d)	42[b]	51[a]	51[a]
Total production [a] (g/l)	8.677[b]	22.50[a]	24.43[a]
Productivity [g/(l·d)]	0.207[b]	0.441[a]	0.479[a]

[a] Five batches, [b] Three batches

TABLE III. NUCLEIC ACID-RELATED COMPOUNDS OBTAINED FROM *C. MILITARIS*

Contents	Extract from mycelia (mg)	Medium (mg)
C. militaris NBRC 9787 (18d)		
Mycelia	3.29×10^4	
Adenine	2.9	2.8
Guanine	18.5	175.2
Uracil	37.1	105.0
Adenosine	91.1	52.6
Guanosine	78.7	47.1
Uridine	106.8	81. 1
Cordycepin	56.2	2.5×10^3
C. militaris G81-3 (24d)		
Mycelia	3.87×10^4	
Adenine	1.6	5.1
Guanine	23.2	271.7
Uracil	29.2	185.2
Adenosine	45.4	132.0
Guanosine	41.3	97.8
Uridine	45.4	122.6
Cordycepin	129.7	4.5×10^3

In every case of repeated batch culture, the better production that was not less than the initial cycle was maintained up to the third cycle, thereafter, they showed lower cordycepin productions. On the medium exchanging day, sampling for both the last day of the succeeding batch and first day (0 day) of the preceding batch were done and between them the cordycepin production was considered as zero. As we mentioned about the mycelial autolysis, it is quite possible to prevent the mycelia from autolysis by the repeated batch culture resulting in an increased production and productivity. The productivity was calculated as the total cordycepin production (g/l) divided by the total time period needed for that. To have the highest productivity, this period is considered up to the day of the last batch after the glucose concentration became below 0.5% of the initial concentration (Table II).

The results of cordycepin production and productivities of control, mutant and mutant with adenosine per batch calculated from the all sum of all the batches were summarized in Table II and Fig. 8. The productivities of the repeated batches were 34%, 86%

and 82% higher than those of batches for control, mutant and mutant with adenosine, respectively; whereas, those of mutant and mutant with adenosine were 113% and 131% higher than that of the control, respectively. The productivity of the mutant increased 9% when the adenosine was added. Additionally, the productivities of batch operations of mutant and mutant with adenosine were 53% and 70% higher than that of the control. Hence, the effectiveness of the repeated batch culture is obvious in both the cases of mutant and control. Although the production of repeated batch culture was higher than that of the batch culture, in a close investigation, it was observed that some parts of the mycelia did not touch the replenished fresh medium. It might be one of the major explainable causes (Fig. 9 a) for not reaching the production a further higher level. As the culture continued for a long time, the mycelia became curvy; also somewhere it sunk into the medium and some parts became cracked, these facts may be considered as the reasons for decreased production of cordycepin (Figs. 9 b, c & d).

In this experiment, it was revealed that the application of repeated batch culture could significantly increase the production and productivity in contrast to the batch culture. Therefore, this technique might be applicable for industrial production of cordycepin for future uses, leading to develop a continuous production protocol. It is also mentionable that it could reduce the labor, time and energy.

3.2 Nucleic acid-related compounds

The contents of nucleic acid-related compounds in the extract from the mycelia and the medium by the batch operation for the mutant and the control are summarized in Table III.

Analyzing the results, it is evident that major portion of cordycepin existed in the medium, that is, the synthesized cordycepin was immediately excreted in the medium. In case of the control, it was found that the concentrations of adenosine and guanosine decreased as the cordycepin concentration increased. On the other hand, in case of the mutant, the concentrations of adenosine and guanosine increased with an increase of cordycepin concentration. This result suggests that the production of cordycepin by mutant is linked to either adenosine and/or guanosine in the medium.

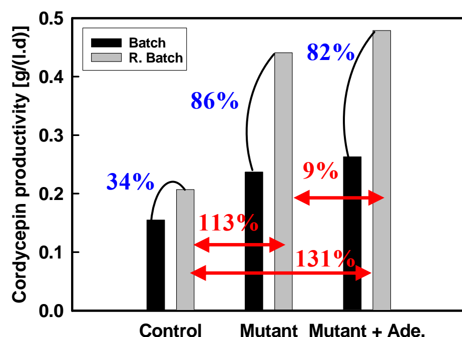

Figure 8. Comparative cordycepin productivities in batch and repeated batch cultures for control, mutant and mutant+adenosine

(a) Untouched mycelia

(b) Curved mycelia

(c) Sunk mycelia

(d) Cracked mycelia

Figure 9. Photographs of untouched, curved, sunk and cracked mycelia in repeated batch culture

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IV. CONCLUSION

The repeated batch culture presently investigated was significantly effective in increasing the productivity of cordycepin. This culture technique might convey valuable information to develop a continuous production protocol for industrial uses. It is necessary to mention that the cordycepin productivities reported by a number of authors [2], [4]-[10] is much lower than that of our present research study [0.48 g/(l·d)] (Table V). The present research will also provide valuable information for mushroom researchers, cancer and radiation biologists, chemical engineers, biotechnologists, medical practitioners, and personnel in the pharmaceutical industries.

TABLE V. CORDYCEPIN PRODUCTIVITIES BY SEVERAL AUTHORS

References	Methodology	Productivity [g/(l·d)]
Mao & Zhong, 2004 [7]	Batch culture	0.015
Mao *et al.*, 2005 [8]	Batch culture	0.019
Mao & Zhong, 2006 [2]	Batch culture	0.025
Masuda *et al.*, 2006 [9]	Batch culture	0.032
Shih *et al.*, 2006 [10]	Batch culture	0.092
Masuda *et al.*, 2007 [4]	Batch culture	0.158
Das *et al.*, 2010 [5]	Batch culture	0.190 (mutant)
		0.102 (control)
Das *et al.*, 2009 [6]	Batch culture + glycine	0.227 (mutant)
	Batch culture + adenosine	0.286 (mutant)
This study	Repeated batch culture (RB)	0.207 (control)
	RB	0.441 (mutant)
	RB + adenosine	0.479 (mutant)

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