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Improved Cordycepin Production in a Liquid Surface Culture of *Cordyceps militaris* Isolated from Wild Strain

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Abstract

A *Cordyceps militaris* NBRC 10352-3 strain that was isolated from *C. militaris* NBRC10352 produced 68 mg of cordycepin from 100 ml of medium, which was the highest level of cordycepin among 60 isolates from three *C. militaris* (NBRC 9787, 100741 and 103752) strains. Interestingly, a liquid surface culture of *C. militaris* NBRC 103752-3 produced 2-fold cordycepin to that in a submerged culture. Cordycepin production was significantly affected by specific surface area (SSA) in the liquid surface culture, and 120.9 mg of cordycepin was produced on SSA of 1.57 cm^{-1} (from 50 ml). The addition of glycine and adenine as an additive to its culture medium was optimized by an experimental design. When 6.75 g/l of adenine was added to the culture, 315.7 mg of cordycepin was produced from 50 ml medium, improving the cordycepin production by 4.7-fold. In this study, the production and productivity of cordycepin were significantly improved in *C. militaris* wild type by a single cell colony isolation and additives without adopting any mutational technologies. This *C. militaris* NBRC 10352-3 strain can be used as a new cordycepin-hyperproducing one, instead of a cordycepin-hyperproducing mutant.

Keywords: *Codyceps militaris*, cordycepin, medicinal mushroom, liquid surface culture, single cell colony isolation

1. Introduction

Cordyceps militaris is an emtomopathogenic Ascomycete and is well-known as a traditional Chinese medicinal mushroom. Its fruiting body has various bioactive compounds, which show anti-tumor or anti-oxidant activities. The mycelia of *C. militaris* also produce cordycepin, a nucleic acid-based antibiotic which is considered as a secondary metabolite with medicinal values as antitumor, anti-metastatic, anti-virus, anti-inflammation, anti-fibrotic, pneumo-protective and anti-microbial properties [1–5].

Cordyceps and *Ophiocordyceps* produce cordycepin, but its biosynthetic pathway has not been revealed. To reveal its biosynthetic pathway in *Cordyceps* and *Ophiocordyceps,* many studies have been carried out. It was deduced from the experiments using radio-labelled adenosine and ribose that cordycepin may be formed by the reduction mechanism similar to the formation of 2'-deoxyadenosine in *C. militaris* [6]. On the other hand, genome and transcriptome analyses were employed for investigation of the biosynthetic pathway [7–9]. Even though many efforts, its biosynthetic pathway in *Cordyceps* and *Ophiocordyceps* are not well understood.

In order to meet the demand, chemical synthesis or extraction from natural *C. militaris* were tried. In the case of chemical synthesis, many troublesome steps have to be carried out [10–12]. In the case of cordycepin extraction from natural *C. militaris*, the natural source of *C. militaris* is limited [1, 13]. To overcome these problems, as an alternative, the fermentation approach to obtain large amount of cordycepin has been investigated [1]. The fermentation technique is promising and useful because *C. militaris* can be cultivated in a flask and jar-fermentor, and also produces cordycepin.

In this study, we tried to improve cordycepin production in a liquid surface culture of *C. militaris* isolated by single cell isolation and increased cordycepin productivity by combining several factors.

2. Materials and Methods

2.1. Microorganism and single cell colony isolation

C. militaris NBRC 9787, NBRC 100741 and NBRC 103752 strains were purchased from Biological Research Center, NITE (NBRC, Tokyo, Japan). The mycelia of each strain were dissolved in 1 ml of medium, which is composed of 5 g/l peptone, 3 g/l yeast extract and 1 g/l $MgSO_4 \cdot 7H_2O$ as a rehydration fluid. The suspension was then transferred to PDA (Nissui Pharmaceutical Co., Ltd. Japan), and incubated for 8 d at 25°C. The PDA plates were used for cultivation in a liquid medium and the PDA slants were stored at 4°C as a stock culture.

Single cell colony isolation from cell suspension was done by a dilution method. Mycelia were diluted serially with a liquid medium, which is composed of 45 g/l yeast extract, 40 g/l glucose, 0.5 g/l KH_2PO_4, 0.5 g/l K_2HPO_4, 0.5 g/l $MgSO_4 \cdot 7H_2O$ and 0.1 g/l $FeSO_4 \cdot H_2O$. Diluted mycelia were plated on a PDA plate, and incubated at 25°C for 8 d. Each isolated colony was further transferred onto the new PDA plates to test cordycepin production. Cordycepin screening was carried out using a 24 deep well plate in a production medium (pH 5.6), composing 72.5 g/l yeast extract, 62.6 g/l glucose and Vogel's medium with 1/10 concentration (0.28 g/l sodium citrate $2H_2O$, 0.50 g/l KH_2PO_4, 0.20 g/l NH_4NO_3, 0.02 g/l $MgSO_4 \cdot 7H_2O$, 0.01 g/l $CaCl_2 \cdot 2H_2O$, 0.46 mg/l citric acid, 0.50

mg/l $ZnSO_4$, 0.1 mg/l Fe $(NH)_4(SO_4)_2 \cdot 6H_2O$ and 0.025 mg/l $CuSO_4 \cdot 5H_2O$) for 24 d at 25°C [14, 15].

2.2. Liquid surface culture of *C. militaris*

The high cordycepin producing *C. militaris* strain was cultivated in a production medium. Cultivation was carried out by inoculating 3 disks (1 cm diameter) of mycelia, grown on PDA plates for 8 d, into 500 ml Erlenmeyer flask containing 100 ml of production medium and incubated at 25°C for 24 d in a surface culture. The culture supernatant was sampled periodically and kept at -30°C for further analysis.

Since culture time is more than 10 d, culture broth is evaporated. Therefore, the cordycepin production was defined as an amount of produced cordycepin during the culture as follow: amount (mg) = cordycepin concentration (mg/ml) × V_0 × (1 – kt), where V_0, k and t denote initial culture volume (ml), evaporation rate (ml/d) and culture time (d), respectively. In this study, k=0.02 ml/d at 25°C.

2.3. Specific surface area (SSA) of liquid surface culture

Effect of SSA on the production of cordycepin was investigated by adjusting culture volumes from 50 ml to 200 ml in Erlenmeyer flask, corresponding SSA from 1.57 cm^{-1} to 0.34 cm^{-1}. The cultures were incubated at 25°C for 24 d and the culture broth was subsequently collected periodically and stored at -30°C before cordycepin analysis.

2.4. Partial purification of cordycepin

Culture broth was boiled for 30 min and then centrifuged at 8000 × g for 30 min at 4°C. The supernatant was mixed with 1 liter of chilled ethanol and left to stand at 4°C overnight. The resultant precipitate was removed by centrifugation at 8000 × g for 30 min. Then the supernatant was mixed with activated charcoal powder and kept at 60°C. After 6 h the sample was filtered, the supernatant was dried in a rotary evaporator. The crude cordycepin was achieved and then added with water and *n*-hexane (1:1). The cordycepin crystal was formed and obtained as a precipitate.

2.5. Analytical methods

The samples were thawed and centrifuged at 15000 × g, 4°C for 10 min. Screening of high cordycepin producing strain in the 24 deep well plates was performed by thin layer chromatography (TLC) with eluent, composing of chloroform: methanol: H_2O = 52: 7: 0.5. The retention factor of cordycepin (R_f) is 0.46. The high-performance liquid chromatography (HPLC) was used for measuring cordycepin concentration as follow. The supernatant was mixed with 2% methanol with 1:1 ratio and filtered through 0.45 μm filter before analysis. The HPLC was equipped with UV detector at 260 nm (Shimadzu, Tokyo, Japan) and TSK-gel ODS-80Ts (Tosoh Corp., Japan) was used at 40°C with UV detector at 260 nm with 0.1% phosphoric acid: methanol with 98:2 ratio (v/v) as a mobile phase [16]. Used cordycepin as a reference standard was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

3. Results and Discussion

3.1. Isolation of high cordycepin producing strain

Twenty single cell-isolated colonies from the mycelia suspension of each *C. militaris* strain (NBRC 9787, 100741 and 103752) were inoculated and cultivated statically using a 24 deep well plate in a production medium at 25°C for 24 d. The amount of cordycepin produced by these 60 colonies are shown in Table S1 (Supplementary information). The highest cordycepin producing colony in *C. militaris* NBRC 9787-3, 100741-13 and 103752-3 strains was cultivated in a liquid surface culture in 500 ml flask containing 100 ml of production medium. The highest cordycepin amount, 68 mg (1.60 g/l), was obtained at the culture time of 21 d in *C. militaris* NBRC103752-3 (Fig. 1). The reproducibility of cordycepin production in *C. militaris* NBRC 103752-3 was confirmed by three independent cultivation (60, 62 and 68 mg, Fig. S1 of supplementary information). Therefore, *C. militaris* NBRC 103752-3 strain was used throughout in this experiment.

Cordycepin production of *C. militaris* NBRC 103752-3 in the liquid surface culture was compared to that in the submerged culture at agitating rate of 125 rpm with 100 ml of production medium. At 13 d, liquid surface and submerged cultures yielded 16 mg and 8 mg, respectively (data not shown). This result indicates that the liquid surface culture is more suitable for cordycepin production than the submerged culture.

3.2. Effect of SSA on cordycepin production in liquid surface culture

For the liquid surface culture of fungi, surface area is important for its growth of mycelia and secondary metabolite production [17]. Therefore, the effect of SSA on cordycepin

production in *C. militaris* NBRC 103752-3 was investigated. In the 500 ml flasks, 4 variables of medium height (0.64, 1.28, 1.92 and 2.56 cm) were adjusted using 50, 100, 150 and 200 ml of medium, respectively. SSA was calculated by dividing surface area (cm^2) with volume (ml) of the medium. When the SAA was 1.57 cm^{-1} the cordycepin amount was 120.89 mg (3.68 g/l) (Fig. 2A). Mycelial growth and cordycepin amount were coincident (Fig. 2B). This suggests that mycelia growth attributed the improvement of cordycepin production. Higher SAA increased dissolved oxygen supply, which was responsible for the increased mycelial growth. Increased mycelial growth increased cordycepin production in the liquid surface culture. On the other hand, even though *C. militaris* cells are cultivated in a submerged culture with sufficient oxygen supply, cordycepin production was less than that cultivated in a liquid surface culture [18, 19]. It suggests that moderate amount of oxygen and less mycelial damage are required for the cordycepin production in *C. militaris*.

3.3. Effect of an additive on cordycepin production

The effect of additives on cordycepin production was investigated in the liquid surface culture of *C. militaris* NBRC 103752-3. Cordycepin is an analogue of adenosine, suggesting that the addition of intermediates in purine metabolism into culture medium may enhance the cordycepin production. The addition of adenine or 2'-deoxyadnosine enhanced cordycepin production by 1.4- and 1.2-fold, respectively (Fig. 3). On the other hand, amino acids, arginine and phenylalanine, inhibited cordycepin production. In a previous report, the level of cordycepin in fruiting body of *C. militaris* was increased by the addition of 18 ppm sodium selenite [20]. However, in this study, the addition of 18

ppm sodium selenite to its culture medium inhibited the cordycepin production (Fig. 3), indicating the metabolism between mycelia and fruiting body is different for its cordycepin production. Gene expression pattern in the cultivated mycelia of *C. militaris* was also different from that in its fruiting body [8]. Addition of glycine and adenine was optimized using D-optimal design analysis, resulting in the additions of 7 g/l adenine and 8 g/l glycine together yielded the highest productivity (315.7 mg, 12.1 g/l) of cordycepin (Table S2 of Supplementary information). However, from the response surface plot for cordycepin production, the addition of glycine was not significant compared to that of adenine for cordycepin production (Fig. 4). One-factor design analysis with two responses was applied for optimizing adenine concentration. Quadratic model of response surface (Table S3 of Supplementary information) led the optimum adenine concentration. When 6.75 g/l of adenine was added, the cordycepin produced amount was 312 mg (12.0 g/l). This model validation was done in 500 ml flasks and the predicted amount was 289 mg (data not shown). D-optimal design analysis and one-factor design analysis have been used for the optimization of microbial growth, protein expression and bioactive compound production in microorganisms [21-24]. These analyses are useful to improve the production of bioactive compounds, secondary metabolites and enzymes in micro-organisms.

Cordycepin has adenine structure in itself, indicating that adenine may be a precursor of cordycepin. In fact, several researchers reported that the addition of adenine adenosine in the surface culture of *C. militaris* improved the cordycepin production [15, 18]. However, the role of adenine and adenosine in its cordycepin biosynthetic pathway has not been clarified yet. Some researchers suggest that ribonucleotide reductase, which catalyzes nucleotide diphosphate (or nucleotide triphosphate) to 2'-deoxynucleotide

diphosphate (or 2'-deoxynucleotide triphosphate), is a candidate to synthesize cordycepin in *C. militaris* [8, 9], but it has not been proved yet. Some analyses of transcriptome and proteome in *C. militaris* including fruiting body formation have been carried out [8, 25] to find out the cordycepin biosynthetic pathway, but still it was not clarified.

Cordycepin productivity of *C. militaris* NBRC 103752-3 in a liquid surface culture was comparable to those in other reports (Table 1). In submerged culture, the cordycepin concentration was lower than 0.4 g/l with productivity of 24.7 mg/l/d, which was coincident with our preliminary culture data. Masuda et el. reported that *C. militaris* NBRC 9787 produced 2.5 g/l of cordycepin for the culture time of 16 d, which is the maximal cordycepin productivity (158 mg/l/d) in the wild type of *C. militaris* strain [15]. They improved the cordycepin production by adopting mutation technology using ion beam irradiation [26] (Table 1). In this work, we improved the cordycepin production without any mutation step, but with only single cell colony isolation, optimization of SSA and adenosine addition in the liquid surface culture, in which its productivity was comparable to that of mutant strain.

3.4. Partial purification of cordycepin

A 348 mg of white powder was obtained from 30 ml of culture broth of the surface culture of *C. militaris* (Fig. 5A). The chromatogram showed that a single peak appeared at the retention times of 11.6 and 11.2 min (Fig. 5B and C) in a cordycepin standard and the partially purified cordycepin from the culture supernatant of *C. militaris* NBRC 103752-3 culture medium, respectively. The purity of partial purified cordycepin was

94.4% (Fig. 5C). The peak of partially purified cordycepin corresponded to that of authentic cordycepin (Fig. 5D).

4. Conclusions

In this study, the improvement of cordycepin production in the liquid surface culture of *C. militaris* was achieved by a single cell colony isolation and the addition of additives to its culture medium. *C. militaris* NBRC103752-3, which was isolated by a single cell colony isolation of *C. militaris* NBRC 103752, finally produced 312 mg of cordycepin from 50 ml of medium supplemented with 6.75 g/l of adenine in a surface culture using a 500 ml flask. The cordycepin productivity (260 mg/l/d) is comparable to that of *C. militaris* mutant. It is possible that *C. militaris* NBRC103752-3 strain can be used as a cordycepin-hyperproducing one without adopting any mutation technologies.

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Figure legends

Fig. 1. Cordycepin production of isolated each *C. militaris* strain. *C. militaris* NBRC 9787-3, 10041-18 and 103752-3, which were isolated from single cell colony process in Table S1 (supplementary information), were cultivated statically in 500 ml flasks with 100 ml of production medium for 24 d. Culture medium in each flask was sampled every 3 d and the amount of cordycepin was measured by HPLC. Open circles: *C. militaris* NBRC 9787-3, open triangles: *C. militaris* NBRC 10041-18, closed circles: *C. militaris* 103752-3. Bars on symbols denote standard deviation (n=3).

Fig. 2. (A) Effect of SSA on the cordycepin production in *C. militaris* NBRC 103752-3. In the same 500 ml flasks, 4 variables of medium height (0.64, 1.28, 1.92 and 2.56 cm) were adjusted using 50, 100, 150 and 200 ml of production medium, respectively. SSA was calculated by dividing surface area (cm^2) with volume (ml) of the medium. Culture medium in each flask was sampled every 3 d and the amount of cordycepin was measured by HPLC. Closed circles: 50 ml medium (SSA is 1.57 cm^{-1}), open circles: 100 ml medium (SSA is 0.77 cm^{-1}), open triangles: 150 ml medium (SSA is 0.47 cm^{-1}), open squares: 200 ml medium (SSA is 0.34 cm^{-1}). Bars on symbols denote standard deviation (n=3). (B) Effect of SAA on cordycepin production and mycelial concentration. Closed circles: cordycepin amount, closed squares: mycelia amount.

Fig. 3. Effect of additives on the cordycepin production in the liquid surface culture of *C. militaris*. All additives except for sodium selenite were added to culture medium at 1 g/l. Concentration of added sodium selenite was 18 mg/l. Bars denote standard deviation (n=3).

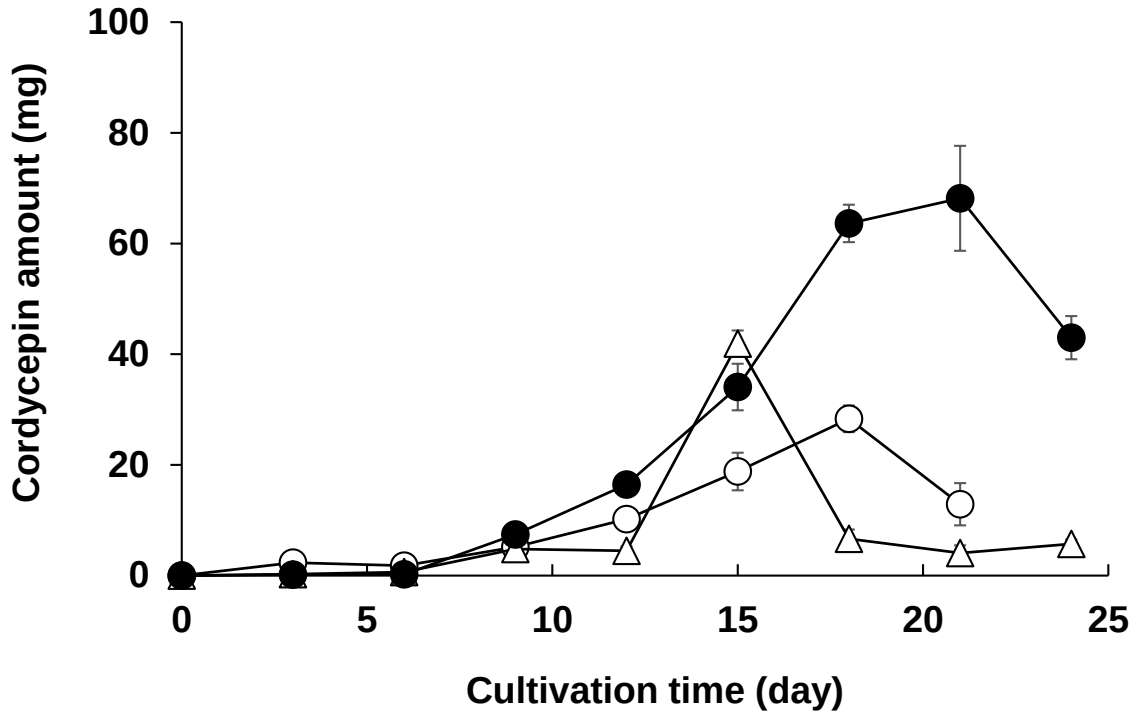
Fig. 4. Three-dimensional response surface plot for cordycepin produced in the liquid surface culture of *C. militaris* as a function of the concentration of adenine and glycine.

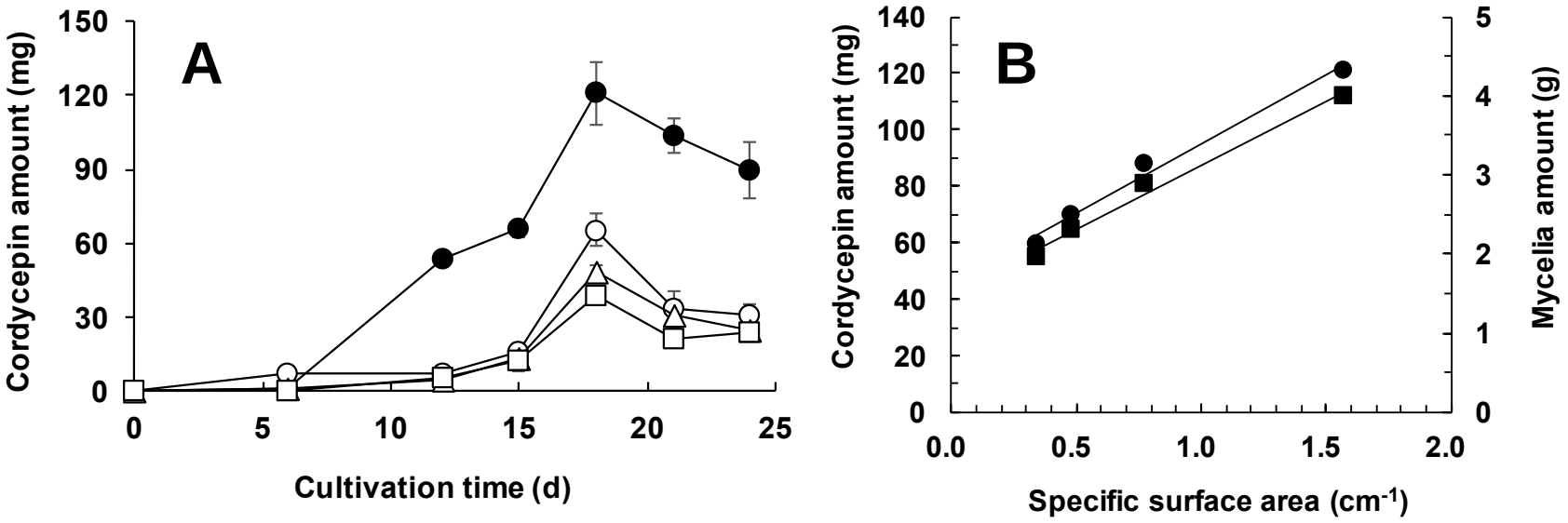
Fig. 5. Partially purified cordycepin powder (A). Chromatogram of authentic cordycepin sample (B), partially purified cordycepin (C) and partially purified cordycepin with authentic cordycepin (D).

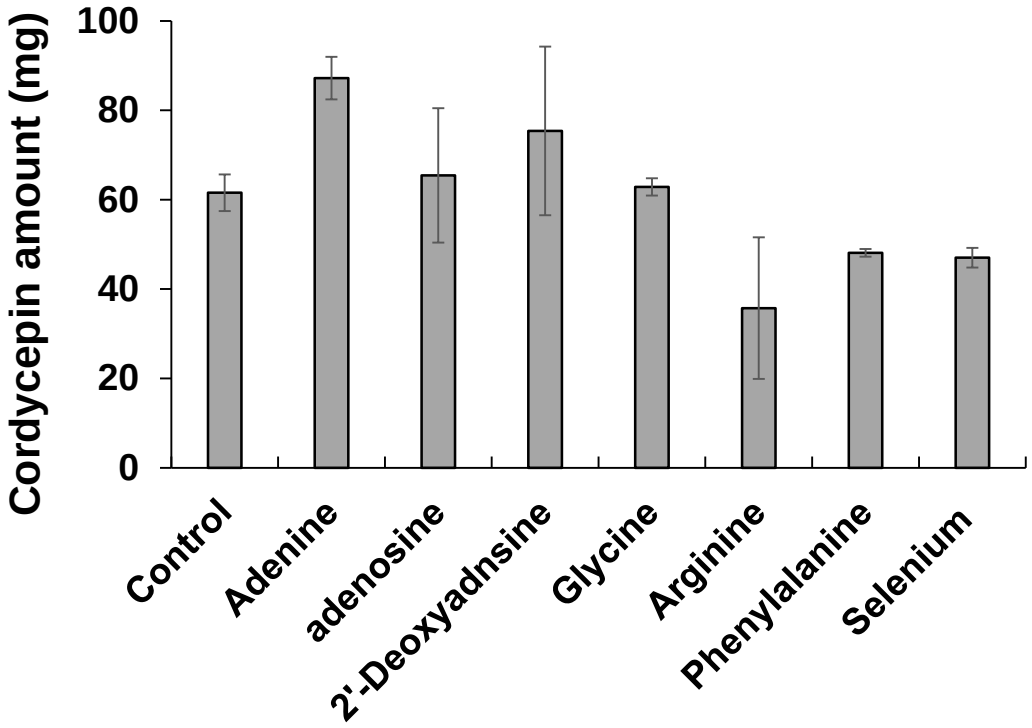
Table 1. Comparison of culture methods

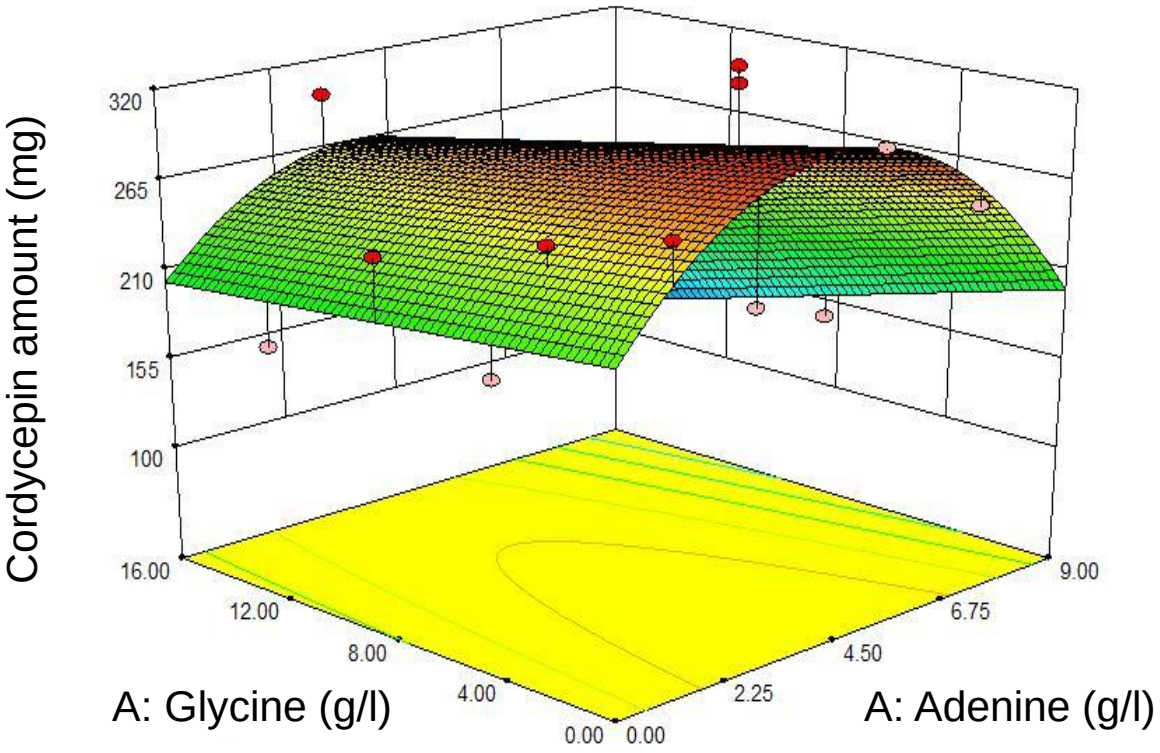
Culture method	Strain	Cordycepin concentration (g/l)	Cordycepin productivity (mg/l/d)	Reference
Submerged culture				
	C. militaris	0.20	15.5	[19]
	C. militaris	0.25	13.6	[27]
	C. militaris	0.42	24.7	[28]
Liquid surface culture				
	C. militaris CCRC 32219	2.20	123	[29]
	C. militaris NBRC 9787	0.64	32.0	[30]
	C. militaris NBRC 9787	2.50	158	[15]
	C. militaris G81-3	8.57	286	[26]
	C. militaris G81-3	14.3	360	[31]
	C. militaris NBRC 103752	312.15 (mg)*	260	This work

* The amount corresponds to 6.24 g/l in 500 ml flask containing 50 ml medium.


Cordycepin amount (mg)
100
80
60
40
20
0
0
5
10
15
20
25
Cultivation time (day)

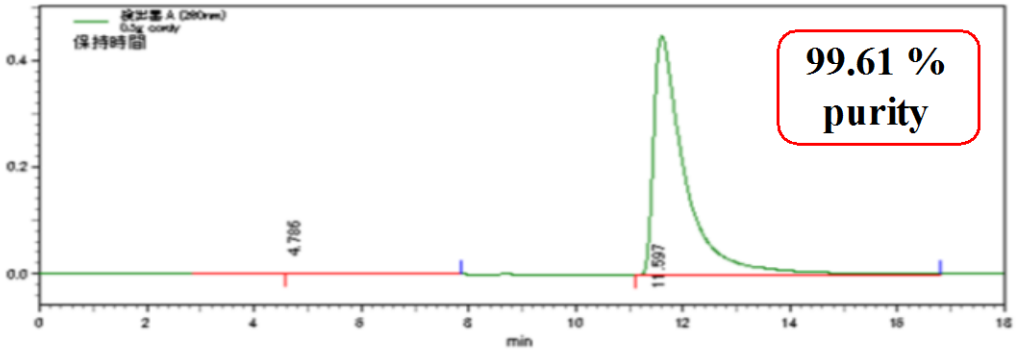

A
Cordycepin amount (mg)
150
120
90
60
30
0
0
5
10
15
20
25
Cultivation time (d)
B
Cordycepin amount (mg)
140
120
100
80
60
40
20
0
0.0
0.5
1.0
1.5
2.0
Specific surface area (cm-1)
Mycelia amount (g)
5
4
3
2
1
0


Cordycepin amount (mg)
100
80
60
40
20
0
Control
Adenine
adenosine
2'-Deoxyadnsine
Glycine
Arginine
Phenylalanine
Selenium

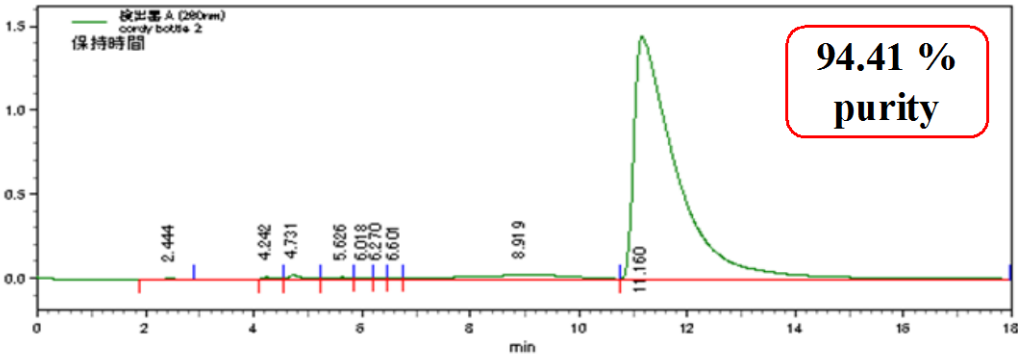

Cordycepin amount (mg)
320
265
210
155
100
16.00
12.00
8.00
4.00
0.00
A: Glycine (g/l)
0.00
2.25
4.50
6.75
9.00
A: Adenine (g/l)

A

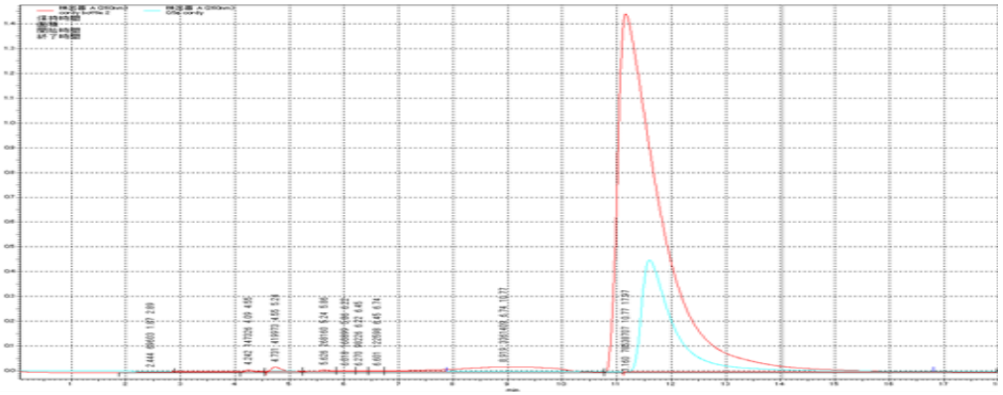
B


保持時間
99.61 %
purity
4.786
11.597
min

C


保持時間
94.41 %
purity
2.444
4.242
4.731
5.626
6.018
6.270
6.601
8.919
11.160
min

D



Supplementary Information

Improved Cordycepin Production in a Liquid Surface Culture of *Cordyceps militaris* Isolated from Wild Strain

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Table S1. Single cell colony isolation of *C. militaris* NBRC 9787, NBRC 100741 and NBRC 103752

Cordycepin Production (g/l)			
Colony number	*C. militaris* NBRC 9787	*C. militaris* NBRC100741	*C. militaris* NBRC 103752
1	0.59	0.41	1.40
2	0.80	0.65	0.45
3	1.54	0.73	1.60
4	0.49	0.16	0.93
5	0.58	0.13	0.40
6	0.22	0.49	0.23
7	0.30	0.31	0.14
8	0.57	0.35	0.19
9	0.46	1.07	0.11
10	0.43	1.31	0.19
11	1.43	1.58	0.05
12	1.13	0.44	0.28
13	0.34	1.67	0.17
14	0.45	1.26	0.14
15	0.35	0.48	0.24
16	0.39	0.25	0.39
17	0.80	1.00	0.49
18	0.81	1.65	0.36
19	0.44	1.39	0.22
20	0.28	0.89	0.26

Table S2. D-optimal design for adenine and glycine as inducer with the response-cordycepin

Run	Adenine (g/l)	Glycine (g/l)	Response Cordycepin (mg)	Predicted value (mg)
1	3	12	249.274	119.59
2	7	8	315.705	209.81
3	9	16	102.516	257.10
4	5	0	311.36	311.55
5	3	12	257.668	241.58
6	0	8	253.101	264.77
7	7	0	264.592	275.85
8	9	8	145.129	159.43
9	3	16	297.824	222.09
10	9	16	101.368	265.12
11	1	0	289.071	275.29
12	7	8	307.942	297.76
13	1	4	269.253	215.95
14	0	12	181.99	119.59
15	0	4	201.855	275.85
16	5	4	201.268	241.58

Table S3. One factor design of adenine with cordycepin and dry weight mycelia as a response in 24 days of cultivation.

Run	Factor 1 A: Adenine (g/l)	Response 1 Cordycepin (mg)	Response 2 Dry mycelia weight (g)
1	0	211.44	2.17
2	0	197.77	2.25
3	4.5	262.3	2.23
4	2.25	243.66	2.23
5	9	274.87	2.72
6	6.75	321.15	2.56
7	9	283.95	2.45

Fig. S1. Cordycepin production of *C. militaris* 103752-3 in a surface culture in 500 ml flasks with 100 ml liquid medium for 24 d. For the reproducibility of cordycepin production of *C. militaris* 103752-3, the same surface culture was performed three times in triplicate. Closed circles: first experiment, Open circles: second experiment, Open triangles: third experiment. Bars on symbols denote standard deviation (n=3).