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Cordycepin production by the potential fungal strains *Cordyceps militaris* BCC 2819 and *Cordyceps cicadae* BCC 19788 in submerged culture during batch and Fed-batch fermentation

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Cordycepin is one of the most important bioactive compounds; the low productivity and long production cycle of cordycepin are barriers to its commercialization. The optimal media for cordycepin production by *Cordyceps militaris* BCC 2819 and *Cordyceps cicadae* BCC 19788, which are potent cordycepin-producing-fungal strains, were determined through statistical experiments. Six nutrients including glucose, adenine, glycine, alanine, casein hydrolysate and vitamin solution were found to influence the cordycepin production by *C. militaris* BCC 2819, while the same factors were found for the cordycepin production by *C. cicadae* BCC 19788 except glucose that was replaced by ammonium sulfate. The highest cordycepin production of 1,176.69 ± 263.33 mg/L was obtained by *C. militaris* BCC 2819 using the central composite design. The highest cordycepin production of 4,259.63 ± 224.20 mg/L was obtained by *C. cicadae* BCC 19788 using the central composite design. Cordycepin production in 5-L fed-batch fermentation by *C. militaris* BCC 2819 and *C. cicadae* BCC 19788 using optimized medium reached maximum production levels of 3,112.50 and 3,587.10 mg/L, respectively, accounted for more than 1.2-fold compared to those in batch fermentation. Furthermore, the highest levels of the bioactive compounds; exopolysaccharide, adenosine and mannitol produced by *C. militaris* BCC 2819 were 43.90 ± 2.51 g/L, 2,897.40 ± 382.47 mg/L and 5,981.10 ± 254.72 mg/L, respectively. The highest levels of the bioactive compounds; exopolysaccharide, adenosine and mannitol produced by *C. cicadae* BCC 19788 were 38.10 ± 2.84 g/L, 3,78520 ± 165.70 mg/L and 6,100.20 ± 191.14 mg/L, respectively. These results demonstrated the new isolates produced high amounts of bioactive compounds, especially cordycepin. Interestingly, this process can be applied for cordycepin production for future applications or scale-up studies.

Key words: Batch fermentation, cordycepin, *Cordyceps militaris*, fed-batch fermentation, *Cordyceps cicadae*, optimization.

INTRODUCTION

Cordycepin (3'-deoxyadenosine), a nucleoside analog, was first isolated from *Cordyceps militaris* (Cunningham et al., 1950). Cordycepin has been used as a medicinal agent due to its anticancer (Yoshikawa et al., 2008), antifungal (Sugar and McCaffrey, 1998), antiviral (Hashimoto and Simizu, 1976), anti-inflammatory, antioxidant (Tuli et al., 2013) and immunological regulation activities (De Silva et al., 2012). Hence, cordycepin has received attention for its potential applications as a functional food and healthcare product. It has been produced by chemical synthesis (Kwon et al., 2003) and microbial fermentation using *C. militaris* (Cunningham et al., 1950) or *Aspergillus nidulans* (Kaczka et al., 1964). However, it is difficult to purify cordycepin produced through chemical synthesis, leading to high production costs. Thus, cordycepin production through microbial fermentation is an interesting avenue.

Microbial fermentation is a cost-effective process for the production of cordycepin on an industrial scale. However, in the present study, cordycepin production through microbial fermentation still needs to be optimized due to its low productivity and long production cycle time upto 40 days (Lim et al., 2012). The production of cordycepin via solid-state fermentation (Wen et al., 2008b) and submerged fermentation (Kang et al., 2012; Wen et al., 2009) has been often used for the cordycepin production, in which solid-state cultivation of fungi on various insect pupae and larvae has been used for commercial purposes (Zhang et al., 2011). Various solid substrates such as rice, oat and wheat were used as solid substrate for cordycepin production (Chen et al., 2010). Moreover, physical condition such as pH, temperature and light condition also affected the cordycepin production using solid state fermentation (Chen et al., 2010; Lim et al., 2012). However, the drawback of solid-state fermentation is that it takes a long time for complete fruiting body development, and it is difficult to achieve commercial scale production (Chen et al., 2010). The consistency of cordycepin composition in the fruiting bodies obtained is one of the key problems for fruiting body production in solid-state fermentation. Presently, liquid static culture (Kang et al., 2014) and submerged fermentation is an alternative process for cordycepin production. Liquid culture is considered as a better culture procedure for industrial purposes because of the shorter cultivation time and the higher cordycepin production yield (Kunhorm et al., 2019). Furthermore, in addition to optimizing the fermentation process, Improving cordycepin-producing fungal strains should also receive attention. Previously, mutant strains that produce higher cordycepin levels than wild-type strains were generated by various technologies, such as ion-beam irradiation (Das et al., 2008) and space mutation treatment (Wen et al., 2008a). The components of the culture medium also affect the cordycepin yield. Some previous research showed that glucose and yeast extract were components that improved cordycepin production in *C. militaris* (Mao et al., 2005; Das et al., 2010). Specific amino acids, precursors, and inducers are also required for secondary metabolite production by microorganisms, including fungi (Oh et al., 2019). Other studies have used different culture components and additives (Wen et al., 2009; Mao and Tu, 2005; Masuda et al., 2007; Das et al., 2009) for cordycepin production in liquid culture. These studies revealed that the most effective components for cordycepin production depend on the fungal strain. Thus, the nutrition requirements and physical conditions, including seed culture preparation, for cordycepin production by microorganisms are of interest. Our previous preliminary study found two fungal strains that produce cordycepin at high levels among those 10 candidate strains chosen (data not shown), *C. militaris* BCC 2819 and *C. cicadae* BCC 19788, and these strains can be used as potential fungal cell factories for high-titer cordycepin production. In this study, the effects of medium components (carbon source, nitrogen source, amino acid and precursors) on cordycepin production were elucidated through Plackett-Burman design. Then, the optimal cordycepin production medium for both fungal strains was determined by central composite design. Validation of the optimized medium was performed in batch fermentation in a 5-L fermenter. Finally, fed-batch fermentation was used to improve cordycepin production and the production of other important bioactive compounds that are effectively synergistic with the mechanism of cordycepin production in animal bodies as functional food supplements.

MATERIALS AND METHODS

Microorganism

C. militaris BCC 2819 and *C. cicadae* BCC 19788 were obtained from Thailand Bioresource Research Center (TBRC). The stock cultures were stored on potato dextrose agar (PDA) slant. The cultures were incubated at 25°C for 5 to 7 days and then supplemented with 20% glycerol. The stock slants were stored at 4°C.

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Table 1. Plackett-Burman design for cordycepin production by *C. militaris* BCC 2819 and *C. cicadae* BCC 19788.

Treatment	Factor										
	A	B	C	D	E	F	G	H	J	K	L
1	40	10	5	5	2	5	10	2	2	4	2
2	20	10	15	2	2	5	20	2	2	1	4
3	40	5	15	5	1	5	20	5	2	1	2
4	20	10	5	5	2	2	20	5	5	1	2
5	20	5	15	2	2	5	10	5	5	4	2
6	20	5	5	5	1	5	20	2	5	4	4
7	40	5	5	2	2	2	20	5	2	4	4
8	40	10	5	2	1	5	10	5	5	1	4
9	40	10	15	2	1	2	20	2	5	4	2
10	20	10	15	5	1	2	10	5	2	4	4
11	40	5	15	5	2	2	10	2	5	1	4
12	20	5	5	2	1	2	10	2	2	1	2
13	30	7.5	10	3.5	1.5	3.5	15	3.5	3.5	2.5	3
14	20	5	15	2	1	2	20	5	5	1	4
15	40	5	5	5	1	2	10	5	5	4	2
16	20	10	5	2	2	2	10	2	5	4	4
17	40	5	15	2	1	5	10	2	2	4	4
18	40	10	5	5	1	2	20	2	2	1	4
19	40	10	15	2	2	2	10	5	2	1	2
20	20	10	15	5	1	5	10	2	5	1	2
21	20	5	15	5	2	2	20	2	2	4	2
22	20	5	5	5	2	5	10	5	2	1	4
23	40	5	5	2	2	5	20	2	5	1	2
24	20	10	5	2	1	5	20	5	2	4	2
25	40	10	15	5	2	5	20	5	5	4	4
26	30	7.5	10	3.5	1.5	3.5	15	3.5	3.5	2.5	3

A =Glucose (g/L), B =peptone (g/L), C =yeast extract (g/L), D =$NH_4(_2SO_4)$ (g/L), E =adenine (g/L), F =glutamine (g/L), G = glycine (g/L), H =alanine (g/L), J =casein hydrolysate (g/L), K =vitamin solution (mL/L), and L =trace solution (mL/L).
Source: Author

Optimization of cordycepin production by *C. militaris* BCC 2819 and *C. cicadae* BCC 19788

The seed culture was prepared in 1,000 mL Erlenmeyer flasks containing 200 mL of potato dextrose broth (PDB) and incubated on a rotary shaker at 200 rpm at 25°C for 5 to 7 days. The culture was blended and transferred by portioned to 50 mL of production medium with 5% v/v.

Optimization of cordycepin production by *C. militaris* BCC 2819 and *C. cicadae* BCC 19788 using Plackett-Burman (PB) design

The PB design (Plackett and Burman, 1946) was used to identify the factors that influenced cordycepin production by *C. militaris* BCC 2819 and *C. cicadae* BCC 19788. A total of 11 factors, that is, glucose, glycine, alanine, glutamate, adenine, casein hydrolysate, peptone, yeast extract, (NH4)2SO4, trace solution (consisting of 14.3 g/L $ZnSO_4.7H_2O$, 2.5 g/L $CuSO_4$, 0.5 g/L $NiCl_2.6H_2O$, 6 g/L $MnCl_2$ and 13.8 g/L $FeSO_4.7H_2O$), and vitamin solution (Blackmore) (Vitamin complex consisted of 75 mg vitamin B1 (thiamine hydrochloride), 10 mg vitamin B2 (riboflavin), 50 mg nicotinamide, 25 mg calcium pantothenate, 10 mg vitamin B6 (pyridoxine hydrochloride), 25 mcg vitamin B12 (cyanocobalamin), 15 mcg biotin, 500 mg vitamin C (derived from ascorbic acid 260 mg and calcium ascorbate 290.5 mg), 10 mg choline bitartrate, 10 mg inositol, 10 mg zinc amino acid chelate (zinc 2 mg), 175 mg calcium phosphate, and 75 mg magnesium phosphate), were evaluated. The base medium component consisted of 0.5 g/L $MgSO_4$, 0.5 g/L KH_2PO_4 and 0.5 g/L K_2HPO_4. The PB experimental design with center points (26 treatments) for cordycepin production by *C. militaris* BCC 2819 and *C. cicadae* BCC 19788 is shown in Table 1. The center points were run to evaluate the curvature and the linearity of the variables. For each condition, the cultures were shaken at 200 rpm on an orbital shaker at 25°C for 11 days. The experiments were performed in triplicate.

Optimization of cordycepin production by *C. militaris* BCC 2819 and *C. cicadae* BCC 19788 using central composite design (CCD)

After screening the influencing factors by PB design, the optimal

value of each influencing factors was optimized using CCD (Box and Wilson, 1992) to enhance cordycepin production. The factor name and actual level of the factors for *C. militaris* BCC 2819 and *C. cicadae* BCC 19788 for each experimental design (47 treatments) are shown in Tables 2 and 3, respectively. The cultures were shaken at 200 rpm on an orbital shaker at 25°C for 11 days. The experiments were performed in triplicate. The adequacy of the quadratic model was determined using the coefficient of determination (R^2) and analysis of variance (ANOVA). Design Expert software version 10.0 (State-Ease, US) was used to draw contour plots to explain the influences of the variables on cordycepin yield and predict the best conditions for cordycepin production by *C. militaris* BCC 2819 and *C. cicadae* BCC 19788. The production in potato dextrose broth (PDB) was used as a control.

Cordycepin production by *C. militaris* BCC 2819 and *C. cicadae* BCC 19788 in batch fermentation

After optimization in shake flasks, cordycepin was produced in 5-L stirred tank fermenters (BIOSTAT B Plus, Sartorius stedim, Germany). A total of 10% seed culture was inoculated into 3 L of optimal medium and cultivated at 25°C with agitating at 400 rpm at an aeration rate of 1 vvm. Fermentation finished after glucose was depleted. Samples were taken to measure the cell mass, exopolysaccharide and active compounds (cordycepin, adenosine and mannitol).

Cordycepin production by *C. militaris* BCC 2819 and *C. cicadae* BCC 19788 in fed-batch fermentation with an exponential feed rate

For fed-batch fermentation, the initial culture was grown in 1.5 L for the batch process. After the culture reached the exponential phase, 2 L of feeding medium was added to the culture according to the feeding profile using the following model (Chongchittapiban et al., 2016):

$$F_0 = \frac{\mu_{set}}{S_0 Y_{X/S}} X_{10} V_0 e^{\mu_{set}(t-t_0)}$$

where μ_{set} = 0.02 h^{-1}, S_0= 150 g. L^{-1}, X= 36.3 g. L^{-1}, and $Y_{X/S}$= 0.67 $g.g^{-1}$ for *C. militaris* BCC 2819, and μ_{set} = 0.02 h^{-1}, S_0= 100 g. L^{-1}, X= 35.36 g. L^{-1}, and $Y_{X/S}$= 0.39 $g.g^{-1}$ for *C. cicadae* BCC 19788.

The feeding medium for *C. militaris* BCC 2819 consisted of glucose 150 g/L, adenine 4.41 g/L, glycine 26.4 g/L, alanine 10 g/L, glutamine 2 g/L, casein hydrolysate 5 g/L, peptone 10 g/L, yeast extract 15 g/L, $(NH_4)_2SO_4$ 2 g/L, $MgSO_4$ 0.5 g/L, KH_2PO_4 0.5 g/L, K_2HPO_4 0.5 g/L, trace element 2 ml/L and vitamin solution 4 ml/L. The feeding medium for *C. cicadae* BCC 19788 consisted of glucose 100 g/L, adenine 5 g/L, glycine 30 g/L, alanine 5 g/L, glutamine 2 g/L, casein hydrolysate 10 g/L, peptone 10 g/L, yeast extract 15 g/L, $(NH_4)_2SO_4$ 5.68 g/L, $MgSO_4$ 0.5 g/L, KH_2PO_4 0.5 g/L, K_2HPO_4 0.5 g/L, trace element 2 mL/L and vitamin solution 6 mL/L.

Dried cell weight and exopolysaccharide quantification

The culture was filtered through filter paper (Whatman), and the mycelium and supernatant were separated. The mycelium was rinsed and lyophilized to obtain the dry cell weight. The supernatant was added to cold 95% ethanol 4 times to precipitate exopolysaccharide. The exopolysaccharide was lyophilized to obtain the dry weight.

Cordycepin and adenosine extraction and quantification

Cordycepin and adenosine were produced inside the cell and secreted into the medium. After filtration, the mycelium was ground with liquid nitrogen to obtain a fine powder. The mycelium powder was extracted with water at 80°C for 3 h to obtain cordycepin and adenosine. The cordycepin and adenosine concentrations were determined by HPLC equipped with a UV detector (Waters 2489 UV/Visible detector, Waters, Massachusetts, US) and reversed-phase column (Xterra MS C18 column, Waters, Massachusetts, US). Methanol (8%) was used as the mobile phase. The flow rate was 0.5 mL/min, and the column temperature was 25°C. The total cordycepin and adenosine contents were measured by quantification of each compound from the supernatant and extracted solution of mycelium and comparing the values to standard curves (10 - 100 mg/L).

Mannitol extraction and quantification

Mannitol was extracted from dried mycelium of both fungal strains. The dried mycelium was ground with liquid nitrogen to obtain a fine powder. The mycelium powder was extracted with 0.1 M phosphate buffer pH 7. The mannitol concentration was determined by HPLC with an RI detector (RI 501, Shodex, Yokohama, Japan) and an ion exchange column (Aminex HPX-87H column, Biorad, California, US). Sulfuric acid (0.025 M) was used as the mobile phase. The flow rate was 0.5 mL/min, and the column temperature was 65°C. The content of mannitol was compared with a standard curve of mannitol ranging from 0.0625 to 20 g/L.

RESULTS

The influential factors of cordycepin production by *C. militaris* BCC 2819 and *C. cicadae* BCC 19788 using Plackett-Burman design

The data listed in Tables 4 and 5 show the variation in dry cell weight, exopolysaccharide and active compounds produced by *C. militaris* BCC 2819 and *C. cicadae* BCC 19788, respectively, from 27 treatments. The dry cell weights and exopolysaccharides produced by *C. militaris* BCC 2819 were 6.59 ± 0.91 - 25.54 ± 1.11 g/L to 0.72 ± 0.17 - 16.60 ± 1.61 g/L, respectively. Additional bioactive compounds such as cordycepin, adenosine and mannitol were produced in the ranges of 64.17 ± 0.46 - 960.14 ± 263.33 L, 49.59 ± 2.21 - 632.51 ± 229.30, and 0 - 224.46± 19.44 mg/L, respectively. The highest cordycepin concentration of 960.14 ± 263.33 mg/L produced by *C. militaris* BCC 2819 was obtained with medium containing glucose 40 g/L, peptone 10 g/L, yeast extract 15 g/L, $(NH_4)_2SO_4$ 2 g/L, adenine 1 g/L, glutamine 2 g/L, glycine 20 g/L, alanine 2 g/L, casein hydrolysate 5 g/L, vitamin solution 4 mL/L, and trace solution 2 mL/L.

Table 2. Central composite design for cordycepin production by *C. militaris* BCC 2819.

Treatment	Factor					
	A	B	C	D	E	F
1	60	5	30	10	5	4
2	60	5	30	10	5	4
3	60	5	30	5	15	4
4	60	5	30	5	15	4
5	60	5	20	10	5	6
6	60	5	20	10	5	6
7	60	2	30	5	15	6
8	60	2	30	5	15	6
9	40	5	20	10	15	6
10	40	5	20	10	15	6
11	60	2	30	10	5	6
12	60	2	30	10	5	6
13	40	5	30	5	5	4
14	40	5	30	5	5	4
15	60	5	20	5	15	6
16	60	5	20	5	15	6
17	60	2	20	10	5	4
18	60	2	20	10	5	4
19	40	2	30	5	5	6
20	40	2	30	5	5	6
21	40	5	20	5	5	6
22	40	5	20	5	5	6
23	60	2	20	5	15	4
24	60	2	20	5	15	4
25	40	2	20	10	15	4
26	40	2	20	10	15	4
27	40	2	30	10	15	6
28	40	2	30	10	15	6
29	40	5	30	10	15	4
30	40	5	30	10	15	4
31	40	2	20	5	5	4
32	40	2	20	5	5	4
33	34.35	3.5	25	7.5	10	5
34	65.65	3.5	25	7.5	10	5
35	50	1.15	25	7.5	10	5
36	50	5.85	25	7.5	10	5
37	50	3.5	17.18	7.5	10	5
38	50	3.5	32.83	7.5	10	5
39	50	3.5	25	3.59	10	5
40	50	3.5	25	11.41	10	5
41	50	3.5	25	7.5	2.18	5
42	50	3.5	25	7.5	17.83	5
43	50	3.5	25	7.5	10	3.44
44	50	3.5	25	7.5	10	6.57
45	50	3.5	25	7.5	10	5
46	50	3.5	25	7.5	10	5
47	50	3.5	25	7.5	10	5

A =Glucose (g/L), B = adenine (g/L), C =glycine (g/L), D =alanine (g/L), E = casein hydrolysate (g/L), and F = vitamin solution (mL/L).
Source: Author

Table 3. Central composite design of cordycepin production by *C. cicadae* BCC 19788.

Treatment	Factor					
	A	B	C	D	E	F
1	15	5	30	15	5	4
2	15	5	30	15	5	4
3	15	5	30	5	10	4
4	15	5	30	5	10	4
5	15	5	20	15	5	6
6	15	5	20	15	5	6
7	15	2	30	5	10	6
8	15	2	30	5	10	6
9	5	5	20	15	10	6
10	5	5	20	15	10	6
11	15	2	30	15	5	6
12	15	2	30	15	5	6
13	5	5	30	5	5	4
14	5	5	30	5	5	4
15	15	5	20	5	10	6
16	15	5	20	5	10	6
17	15	2	20	15	5	4
18	15	2	20	15	5	4
19	5	2	30	5	5	6
20	5	2	30	5	5	6
21	5	5	20	5	5	6
22	5	5	20	5	5	6
23	15	2	20	5	10	4
24	15	2	20	5	10	4
25	5	2	20	15	10	4
26	5	2	20	15	10	4
27	5	2	30	15	10	6
28	5	2	30	15	10	6
29	5	5	30	15	10	4
30	5	5	30	15	10	4
31	5	2	20	5	5	4
32	5	2	20	5	5	4
33	2.18	3.5	25	10	7.5	5
34	17.83	3.5	25	10	7.5	5
35	10	1.15	25	10	7.5	5
36	10	5.85	25	10	7.5	5
37	10	3.5	17.18	10	7.5	5
38	10	3.5	32.83	10	7.5	5
39	10	3.5	25	2.18	7.5	5
40	10	3.5	25	17.83	7.5	5
41	10	3.5	25	10	3.59	5
42	10	3.5	25	10	11.41	5
43	10	3.5	25	10	7.5	3.44
44	10	3.5	25	10	7.5	6.57
45	10	3.5	25	10	7.5	5
46	10	3.5	25	10	7.5	5
47	10	3.5	25	10	7.5	5

A =$NH_4(_2SO_4)$ (g/L), B = adenine (g/L), C = glycine (g/L), D = alanine (g/L), E = casein hydrolysate (g/L), and F = vitamin solution (mL/L).
Source: Author

Table 4 . Cordycepin, adenosine, mannitol, dry cell weight and exopolysaccharide produced by *C .militaris* BCC 2819 using the Plackett-Burman design.

Treatment	Cordycepin (mg/L)	Adenosine (mg/L)	Mannitol (mg/L)	Dry cell weight (g/L)	Exopolysaccharide (g/L)
1	436.77 ± 306.17	367.28 ± 222.77	40.92 ± 16.44	17.78 ± 2.47	4.49 ± 1.61
2	126.28 ± 69.39	129.28 ± 62.90	1.13 ± 1.00	11.15 ± 0.24	4.65 ± 2.42
3	586.97 ± 190.50	166.84 ± 59.62	86.69 ± 112.87	22.34 ± 2.61	5.85 ± 2.59
4	81.34 ± 40.82	131.33 ± 15.23	13.48 ± 22.11	10.57 ± 1.33	16.60 ± 1.61
5	84.69 ± 24.64	60.88 ± 14.06	0.86 ± 1.49	9.11 ± 0.44	5.99 ± 0.46
6	64.17 ± 0.46	52.37 ± 13.69	8.05 ± 9.23	9.39 ± 1.15	4.36 ± 1.24
7	515.50 ± 280.89	282.13 ± 57.11	4.69 ± 4.70	19.26 ± 2.84	7.61 ± 3.12
8	664.96 ± 158.23	170.27 ± 149.92	78.81 ± 36.73	19.24 ± 1.07	3.85 ± 0.00
9	960.14 ± 263.33	145.69 ± 23.46	104.10 ± 32.58	25.54 ± 1.11	7.41 ± 2.33
10	85.65 ± 35.78	49.59 ± 2.21	1.25 ± 1.09	12.09 ± 1.55	6.91 ± 0.57
11	581.58 ± 441.30	632.51 ± 229.31	224.46 ± 19.45	22.49 ± 3.44	7.04 ± 2.43
12	57.39 ± 13.70	64.43 ± 10.46	0.83 ± 1.44	8.56 ± 0.13	2.49 ± 0.61
13	135.94 ± 4.87	185.96 ± 29.09	0.00 ± 0.00	13.28 ± 1.42	5.48 ± 0.47
14	119.82 ± 63.93	123.27 ± 70.40	1.77 ± 3.06	12.51 ± 1.17	7.87 ± 2.34
15	350.06 ± 74.83	321.04 ± 98.62	57.00 ± 22.75	16.93 ± 1.80	3.13 ± 0.35
16	81.21 ± 50.64	70.04 ± 41.85	0.00 ± 0.00	10.50 ± 0.50	7.79 ± 2.09
17	316.86 ± 63.98	520.90 ± 140.13	21.18 ± 31.11	21.10 ± 0.49	4.95 ± 0.54
18	306.68 ± 39.00	351.16 ± 42.13	20.29 ± 26.57	17.01 ± 1.12	7.85 ± 1.80
19	225.20 ± 115.07	386.23 ± 177.87	23.78 ± 26.19	19.61 ± 5.93	8.20 ± 1.56
20	77.25 ± 27.85	65.62 ± 12.19	11.33 ± 8.27	10.73 ± 2.05	9.97 ± 2.95
21	90.20 ± 19.60	86.07 ± 37.07	0.04 ± 0.06	10.13 ± 0.58	16.45 ± 1.62
22	70.50 ± 27.81	118.61 ± 63.97	0.00 ± 0.00	9.51 ± 2.38	7.32 ± 1.24
23	524.86 ± 286.77	317.25 ± 131.11	180.51 ± 13.16	19.78 ± 3.52	4.86 ± 1.11
24	53.91 ± 11.11	51.56 ± 12.01	0.00 ± 0.00	11.01 ± 2.17	6.07 ± 0.88
25	615.90 ± 402.84	493.67 ± 193.76	95.59 ± 3.02	25.38 ± 2.23	8.10 ± 1.13
26	158.89 ± 150.74	157.50 ± 122.90	3.75 ± 5.30	14.43 ± 1.78	5.29 ± 0.04
Control (PDB)	69.57 ± 12.30	107.28 ± 66.31	24.31 ± 26.97	6.59 ± 0.91	0.72 ± 0.17

Source: Author

The dry cell weights and exopolysaccharides produced by *C. cicadae* BCC 19788 were in the ranges of 4.68 ±0.58 - 32.09 ±1.64 g/L and 1.59 ± 0.60 - 27.57 ± 3.18 g/L, respectively.

Cordycepin, adenosine and mannitol were produced in the ranges of 39.47 ± 10.05 – 3,833.39 ± 194.99, 198.24 ± 59.22 – 5,354.24 ± 1,650.152, and 8.53 ± 7.89 - 639.57 ± 209.87 mg/L, respectively.

The highest cordycepin concentration of 3,833.39 ± 194.99 mg/L produced by *C. cicadae* BCC 19788 was obtained with medium containing glucose 20 g/L, peptone 10 g/L, yeast extract 15 g/L, $(NH_4)_2SO_4$ 5 g/L, adenine 1 g/L, glutamine 2 g/L, glycine 10 g/L, alanine 5 g/L, casein hydrolysate 2 g/L, vitamin solution 4 mL/L, and trace solution 4 mL/L.

The factors that influenced cordycepin production were chosen based on the *p* value of ANOVA analyses (Tables S1 and S2) and the generated models were significant for both fungal strains of each factor and the interaction with the other factors (data not shown).

The factors that influenced cordycepin production by *C. militaris* BCC 2819 were glucose, adenine, glycine, alanine, casein hydrolysate and vitamin solution. However, $(NH_4)_2SO_4$, adenine, glycine, alanine, casein hydrolysate and vitamin solution showed a significant effect on cordycepin production by *C. cicadae* BCC 19788. These influential factors were then used in a CCD experiment to determine the optimal level of each factor for cordycepin production by both fungal strains.

Optimization of cordycepin production by *C. militaris* BCC 2819 and *C. cicadae* BCC 19788 using Central Composite Design (CCD)

The CCD was used to optimize the cordycepin production

Table 5. Cordycepin, adenosine, mannitol, dry cell weight and exopolysaccharide produced by *C. cicadae* BCC 19788 using the Plackett-Burman design.

Treatment	Cordycepin (mg/L)	Adenosine (mg/L)	Mannitol (mg/L)	Dry cell weight (g/L)	Exopolysaccharide (g/L)
1	2652.97 ± 936.86	3048.95 ± 851.66	565.64 ± 35.35	24.69 ± 2.46	9.67 ± 2.13
2	2651.36 ± 1126.69	2169.15 ± 998.25	177.24 ± 9.02	14.82 ± 4.43	27.57 ± 3.18
3	3587.73 ± 928.72	5290.52 ± 1348.47	579.16 ± 79.51	31.01 ± 0.92	15.15 ± 3.84
4	3225.67 ± 644.45	1287.97 ± 376.51	17.67 ± 2.97	10.24 ± 1.18	18.81 ± 3.21
5	3359.56 ± 509.26	2589.56 ± 951.67	172.21 ± 22.02	14.94 ± 1.04	15.40 ± 2.36
6	3374.81 ± 463.38	1216.68 ± 416.04	140.69 ± 27.02	12.26 ± 1.56	18.35 ± 0.79
7	2874.20 ± 1350.61	3707.89 ± 922.61	436.66 ± 9.21	24.65 ± 0.67	12.76 ± 1.58
8	2087.15 ± 693.91	3461.37 ± 448.92	15.37 ± 7.78	30.43 ± 2.49	6.88 ± 4.91
9	2117.58 ± 1068.58	4270.68 ± 846.81	420.73 ± 88.68	30.17 ± 0.56	10.84 ± 3.34
10	3833.39 ± 194.99	2113.78 ± 443.83	208.21 ± 36.23	17.71 ± 1.07	13.07 ± 1.74
11	2817.55 ± 1288.25	5354.24 ± 1650.15	639.57 ± 209.87	31.79 ± 4.39	11.10 ± 5.81
12	2778.59 ± 171.64	1016.35 ± 159.04	140.73 ± 10.46	9.19 ± 0.56	10.20 ± 1.46
13	2286.70 ± 712.64	3124.42 ± 1150.89	257.37 ± 45.96	21.73 ± 2.76	12.45 ± 1.95
14	1832.28 ± 1153.75	1374.16 ± 760.75	280.80 ± 49.78	14.52 ± 3.34	21.32 ± 1.47
15	2727.57 ± 1308.77	3072.36 ± 2247.79	55.27 ± 33.76	26.29 ± 1.94	5.61 ± 1.69
16	2782.55 ± 538.44	900.40 ± 286.80	135.56 ± 80.41	10.32 ± 0.75	9.01 ± 3.01
17	1483.35 ± 388.88	2942.04 ± 635.95	443.06 ± 12.53	29.52 ± 0.88	6.45 ± 1.02
18	1718.32 ± 868.76	2278.82 ± 1074.74	603.00 ± 17.34	27.76 ± 1.16	6.39 ± 3.56
19	1343.27 ± 150.73	2838.69 ± 1027.41	489.73 ± 110.39	32.09 ± 1.64	6.41 ± 3.00
20	3711.14 ± 460.30	2404.74 ± 412.39	238.67 ± 15.99	19.57 ± 0.54	12.64 ± 0.37
21	2457.43 ± 352.56	1397.20 ± 231.25	19.15 ± 8.78	12.57 ± 2.42	16.51 ± 0.89
22	3095.82 ± 83.73	1144.86 ± 780.28	104.23 ± 5.13	8.89 ± 1.41	13.73 ± 1.37
23	2379.85 ± 71.15	2769.87 ± 1353.30	469.55 ± 184.74	26.00 ± 2.12	7.13 ± 4.99
24	2674.23 ± 229.71	1308.58 ± 296.71	8.53 ± 7.89	12.27 ± 1.31	15.98 ± 3.95
25	2356.82 ± 768.36	4667.31 ± 1961.86	420.65 ± 86.74	30.48 ± 3.51	20.22 ± 6.74
26	2185.49 ± 147.99	2391.68 ± 264.87	458.55 ± 45.37	17.44 ± 0.33	13.13 ± 2.23
Control (PDB)	39.47 ± 10.05	198.24 ± 59.22	154.54 ± 59.93	4.68 ± 0.58	1.59 ± 0.60

Source: Author

medium for *C. militaris* BCC 2819 and *C. cicadae* BCC 19788. The data listed in Tables 6 and 7 show the variation in dry cell weight, exopolysaccharide, cordycepin, adenosine, and mannitol produced by *C. militaris* BCC 2819 and *C. cicadae* BCC 19788, respectively. The dry cell weights and exopolysaccharides produced by *C. militaris* BCC 2819 were in the ranges of 6.88 ± 0.91 - 25.91 ± 1.11 and 0.84 ± 0.31 - 13.22 ±1.61 g/L, respectively. Cordycepin, adenosine and mannitol were produced in the ranges of 71.25 ± 4.06 – 1,176.69 ± 263.33, 13.34 ± 2.21 – 1,273.52 ± 229.30, and 0.16 ± 0.05 – 1,376.92 ± 122.84 mg/L, respectively. The dry cell weight and exopolysaccharide produced by *C. cicadae* BCC 19788 were in the ranges of 2.84 ± 0.58 - 19.67 ± 1.64 and 4.96 ± 1.52 - 42.4 ± 2.52 g/L, respectively. Cordycepin, adenosine and mannitol were produced in the ranges of 42.58 ± 12.57- 4,259.63 ± 224.20, 206.84 ± 114.52- 6,646.58 ± 242.24, and 147.51 ± 17.08– 1,758.81 ± 43.21 mg/L, respectively.

The results from both strains were analyzed and are shown in Tables S3 and S4, in which the generated models of both fungal strains were significant without lack of fits. The cordycepin production model of *C. militaris* BCC 2819 is shown in Table S3. The *p* value ($p \leq 0.001$) for the model and for the lack of fit ($p = 0.21$) demonstrated that the experimental data fit well with the model. The model showed a determination coefficient value (R^2) of 0.94 for cordycepin production, indicating that the model could explain up to 94% of the observed variation in the response. The equation that correlated the six factors and cordycepin production level by *C. militaris* BCC 2819 was as follows:

Y = 547.71 + 167.50A + 45.09B - 29.98C + 82.35D - 129.50E + 32.74F - 32.90AB + 11.93AC - 78.86AD - 0.98AE + 30.33AF + 20.38 BC + 66.73BD - 51.46BE -

Table 6. Cordycepin, adenosine, mannitol, dry cell weight and exopolysaccharide produced by *C .militaris* BCC 2819 using the central composite design.

Treatment	Cordycepin (mg/L)	Adenosine (mg/L)	Mannitol (mg/L)	Dry cell weight (g/L)	Exopolysaccharide (g/L)
1	756.99 ± 79.48	356.72 ± 25.84	207.74 ± 19.97	22.94 ± 4.57	3.60 ± 1.25
2	1176.69 ± 263.33	611.25 ± 97.48	222.61 ± 23.54	23.67 ± 1.37	6.82 ± 2.14
3	625.20 ± 141.41	428.52 ± 68.41	158.75 ± 14.02	19.05 ± 3.91	0.00 ± 0.00
4	574.11 ± 135.85	358.74 ± 105.77	125.90 ± 20.14	18.49 ± 1.46	0.00 ± 0.00
5	458.92 ± 229.46	343.10 ± 62.14	78.52 ± 15.24	19.74 ± 1.06	5.04 ± 1.54
6	537.70 ± 268.85	334.62 ± 48.25	318.04 ± 58.14	21.14 ± 3.28	4.96 ± 0.94
7	467.03 ± 26.62	177.25 ± 14.28	38.80 ± 3.25	20.79 ± 0.90	4.58 ± 1.17
8	445.06 ± 25.37	200.79 ± 9.74	210.13 ± 4.85	21.16 ± 0.40	5.46 ± 1.08
9	261.75 ± 104.70	124.97 ± 16.72	24.21 ± 3.47	14.63 ± 0.60	6.58 ± 2.04
10	306.51 ± 78.16	203.35 ± 25.47	30.18 ± 4.96	16.00 ± 3.47	6.32 ± 1.85
11	735.77 ± 272.23	714.27 ± 85.46	262.67 ± 5.41	19.92 ± 1.08	4.50 ± 1.74
12	682.88 ± 187.79	300.01 ± 87.54	226.12 ± 15.24	22.52 ± 4.31	1.74 ± 0.94
13	218.20 ± 40.37	94.13 ± 37.14	9.71 ± 1.05	13.40 ± 0.70	5.04 ± 1.06
14	317.47 ± 65.24	201.70 ± 74.57	30.62 ± 10.54	15.11 ± 0.56	8.94 ± 2.54
15	406.25 ± 117.81	185.14 ± 41.35	261.11 ± 39.57	20.15 ± 0.81	3.70 ± 1.21
16	506.84 ± 146.98	434.29 ± 47.58	227.36 ± 58.14	20.87 ± 1.48	4.68 ± 1.06
17	232.93 ± 67.55	91.76 ± 25.25	6.62 ± 2.41	20.51 ± 3.04	3.98 ± 1.24
18	388.70 ± 112.72	280.31 ± 36.85	4.76 ± 3.54	18.60 ± 1.72	5.40 ± 2.24
19	123.16 ± 61.58	26.13 ± 8.48	9.11 ± 4.12	11.40 ± 1.04	4.74 ± 0.93
20	136.23 ± 32.08	71.43 ± 4.14	1.07 ± 1.01	12.98 ± 1.14	4.34 ± 1.34
21	117.00 ± 27.55	47.28 ± 10.47	0.16 ± 0.05	11.77 ± 2.96	2.66 ± 0.57
22	223.29 ± 52.58	99.33 ± 13.74	4.84 ± 1.24	13.71 ± 0.11	1.98 ± 0.47
23	573.34 ± 135.02	433.56 ± 140.80	148.87 ± 15.24	18.74 ± 2.41	1.62 ± 0.62
24	428.68 ± 234.34	147.83 ± 7.85	178.43 ± 14.15	21.64 ± 5.93	3.94 ± 1.27
25	161.71 ± 70.86	55.03 ± 9.20	4.54 ± 2.17	13.75 ± 2.60	7.88 ± 2.07
26	151.85 ± 45.92	59.36 ± 7.11	55.03 ± 9.87	14.18 ± 1.18	3.50 ± 1.34
27	150.63 ± 35.32	53.87 ± 4.73	12.30 ± 5.57	13.53 ± 1.29	7.74 ± 2.54
28	202.36 ± 71.11	90.59 ± 8.49	65.88 ± 13.53	14.13 ± 0.26	13.06 ± 2.67
29	255.50 ± 36.66	105.04 ± 15.47	381.76 ± 24.95	13.72 ± 3.00	11.20 ± 2.37
30	435.91 ± 183.08	324.81 ± 35.47	198.92 ± 57.84	14.95 ± 0.68	9.30 ± 1.93
31	129.52 ± 47.99	13.34 ± 2.21	5.77 ± 2.41	11.34 ± 1.83	2.56 ± 0.85
32	138.36 ± 32.58	50.22 ± 18.44	5.18 ± 3.14	13.15 ± 2.59	4.70 ± 1.32
33	190.81 ± 81.10	78.94 ± 17.08	18.67 ± 3.41	11.79 ± 0.43	8.48 ± 1.86
34	715.11 ± 157.32	403.00 ± 35.85	333.38 ± 24.74	23.94 ± 1.02	3.02 ± 1.28
35	213.34 ± 47.47	114.52 ± 21.00	2.97 ± 1.65	17.43 ± 1.95	3.36 ± 0.68
36	354.48 ± 78.87	336.57 ± 23.32	12.38 ± 4.58	17.64 ± 1.39	13.22 ± 1.61
37	247.73 ± 55.12	111.78 ± 16.25	6.65 ± 2.87	18.44 ± 1.24	1.78 ± 0.47
38	153.89 ± 34.24	59.69 ± 11.02	9.19 ± 4.24	17.08 ± 3.70	5.24 ± 1.26
39	655.23 ± 177.62	645.30 ± 58.25	302.01 ± 58.74	21.15 ± 1.81	11.78 ± 2.59
40	912.99 ± 184.11	607.97 ± 85.24	574.97 ± 124.02	23.53 ± 3.08	12.66 ± 3.07
41	895.54 ± 217.92	1273.52 ± 229.30	392.92 ± 102.14	18.59 ±1.37	9.16 ± 2.54
42	490.18 ± 174.02	207.58 ± 20.19	1376.92 ± 122.84	25.91 ± 1.11	7.64 ± 2.38
43	472.17 ± 33.05	213.68 ± 6.88	315.95 ± 95.89	22.07 ± 2.07	8.44 ± 3.41
44	574.64 ± 137.32	313.82 ± 60.14	298.50 ± 85.47	20.67 ± 1.52	8.40 ± 2.97
45	552.91 ± 185.57	240.66 ± 22.54	495.10 ± 58.47	19.01 ± 3.36	8.60 ± 2.45
46	502.21 ± 64.78	309.07 ± 15.47	464.39 ± 72.47	20.19 ± 1.42	8.50 ± 2.27
47	430.21 ± 55.50	242.16 ± 19.85	452.59 ± 63.25	19.78 ± 5.34	7.20 ± 2.64
Control (PDB)	71.25 ± 4.06	102.48 ± 8.71	25.47 ± 12.02	6.88 ± 0.91	0.84 ± 0.31

Source: Author

Table 7. Cordycepin, adenosine, mannitol, dry cell weight and exopolysaccharide produced by *C. cicadae* BCC 19788 using the central composite design.

Treatment	Cordycepin (mg/L)	Adenosine (mg/L)	Mannitol (mg/L)	Dry cell weight (g/L)	Exopolysaccharide (g/L)
1	1832.85 ± 433.23	1659.27 ± 412.29	433.23 ± 68.27	11.10 ± 0.99	28.48 ± 2.35
2	2853.09 ± 287.97	2931.86 ± 332.23	287.97 ± 24.59	12.27 ± 1.37	29.66 ± 1.78
3	1285.89 ± 333.19	1094.25 ± 187.42	333.19 ± 20.83	13.75 ± 1.57	29.74 ± 2.30
4	1881.09 ± 324.88	2087.04 ± 411.23	324.88 ± 110.52	14.29 ± 0.85	34.68 ± 2.34
5	2853.00 ± 291.80	2608.88 ± 76.18	291.80 ± 38.90	11.59 ± 0.53	24.54 ± 3.24
6	3558.90 ± 316.13	3073.18 ± 295.04	316.13 ± 22.48	8.97 ± 0.25	29.36 ± 3.00
7	2409.86 ± 277.35	3513.97 ± 312.41	277.35 ± 38.72	17.36 ± 2.16	26.92 ± 8.02
8	2253.22 ± 483.41	2510.15 ± 226.89	483.41 ± 59.46	17.21 ± 3.20	35.30 ± 4.20
9	2563.85 ± 303.88	2491.70 ± 204.43	303.88 ± 27.08	12.97 ± 3.74	31.88 ± 1.29
10	2908.25 ± 307.81	2899.04 ± 187.42	307.81 ± 110.52	13.57 ± 1.10	27.50 ± 1.78
11	3648.77 ± 316.05	3088.36 ± 341.82	316.05 ± 9.59	8.80 ± 1.01	36.80 ± 4.33
12	3653.10 ± 355.99	3452.58 ± 293.88	355.99 ± 11.83	8.26 ± 4.11	29.22 ± 4.72
13	4131.80 ± 284.24	3078.85 ± 170.99	284.24 ± 57.62	9.78 ± 1.90	26.54 ± 1.86
14	4259.63 ± 224.20	6646.58 ± 242.24	180.87 ± 65 .88	10.87 ± 3.74	32.24 ± 5.62
15	2517.58 ± 343.73	2999.62 ± 147.18	343.73 ± 12.82	8.50 ± 3.33	24.02 ± 5.81
16	1537.28 ± 320.19	1512.47 ± 216.35	320.19 ± 25.98	9.47 ± 2.81	28.22 ± 2.50
17	1522.70 ± 289.18	1718.21 ± 515.60	289.18 ± 9.58	16.15 ± 4.83	30.20 ± 3.35
18	2033.61 ± 330.80	2290.91 ± 102.80	333.80 ± 16.50	17.10 ± 6.79	27.82 ± 7.58
19	1799.21 ± 340.81	1889.28 ± 222.59	340.81 ± 29.56	13.05 ± 5.35	33.32 ± 6.54
20	1908.30 ± 222.49	2066.72 ± 317.04	222.49 ± 31.42	12.76 ± 5.59	34.96 ± 6.07
21	1508.98 ± 318.86	1668.58 ± 394.18	318.86 ± 78.52	9.99 ± 0.95	27.14 ± 5.92
22	2532.24 ± 517.79	3981.52 ± 353.75	517.79 ± 48.53	9.09 ± 1.98	25.34 ± 2.31
23	3938.12 ± 233.04	3732.03 ± 276.92	233.04 ± 79.42	13.94 ± 7.37	29.46 ± 6.10
24	1784.90 ± 76.18	1786.33 ± 239.94	76.18 ± 6.41	19.67 ± 1.64	29.94 ± 1.14
25	2604.81 ± 392.75	2372.19 ± 340.94	392.75 ± 10.51	16.15 ± 1.51	24.36 ± 3.82
26	2368.54 ± 167.92	3018.80 ± 248.50	167.92 ± 6.85	15.77 ± 6.64	22.78 ± 2.09
27	1142.12 ± 335.29	1587.53 ± 414.00	335.29 ± 48.71	12.98 ± 3.24	34.06 ± 3.12
28	3064.54 ± 307.98	2516.98 ± 305.87	307.98 ± 42.11	15.31 ± 1.43	29.40 ± 4.53
29	2829.10 ± 267.60	2654.60 ± 359.41	267.60 ± 132.04	10.14 ± 1.27	32.66 ± 6.22
30	2865.42 ± 293.23	2490.21 ± 234.41	293.23 ±86.47	10.73 ± 0.75	33.58 ± 3.36
31	3101.15 ± 492.29	2645.72 ± 267.62	492.29 ± 78.52	13.30 ± 4.22	23.34 ± 5.38
32	1296.25 ± 362.00	1410.11 ± 320.81	362.00 ± 72.15	14.75 ± 0.79	23.88 ± 4.25
33	2180.30 ± 1758.81	2406.75 ± 189.10	1758.81 ± 43.21	11.51 ± 1.83	29.38 ± 2.82
34	2267.07 ± 310.97	2525.89 ± 222.58	310.97 ± 31.52	11.02 ± 2.03	31.72 ± 4.73
35	1612.73 ± 414.89	1158.02 ± 206.29	414.89 ± 46.18	13.01 ± 3.44	18.36 ± 5.36
36	2331.27 ± 243.41	2533.30 ± 362.00	243.41 ± 41.20	10.47 ± 5.43	29.76 ± 1.12
37	2211.01 ± 1154.35	2080.00 ± 226.97	1154.35 ± 78.25	10. ± 0.07	25.24 ± 1.02
38	1916.76 ± 344.95	2159.65 ± 162.23	344.95 ± 57.43	12.11 ± 0.34	42.40 ± 2.52
39	1474.06 ± 306.82	1309.83 ± 210.97	306.82 ± 27.13	10.38 ± 3.94	32.02 ± 11.20
40	3253.62 ± 394.43	2637.07 ± 324.60	394.43 ± 45.40	10.31 ± 2.65	26.44 ± 2.39
41	863.43 ± 295.05	1394.57 ± 176.92	295.05 ± 48.16	8.14 ± 2.54	27.90 ± 7.21
42	1412.47 ± 399.66	1858.89 ± 308.91	399.66 ± 26.98	12.71 ± 0.74	35.22 ± 15.24
43	1973.13 ± 468.89	2236.15 ± 158.24	468.89 ± 96.58	11.90 ± 2.25	29.62 ± 3.19
44	3236.59 ± 253.05	3264.85 ± 256.19	253.05 ± 83.23	12.41 ± 6.22	27.80 ± 4.10
45	1706.80 ± 326.75	1948.98 ± 344.18	326.75 ± 79.20	11.24 ± 3.22	28.16 ± 9.41
46	3123.80 ± 261.24	2795.53 ± 498.95	261.24 ± 67.58	11.64 ± 1.50	29.42 ± 7.19
47	213.71 ± 111.27	2252.84 ± 259.56	311.27 ± 100.11	10.99± 2.35	31.04 ± 9.21
Control (PDB)	42.58 ± 12.57	206.84 ± 114.52	147.51 ± 17.08	2.84 ± 0.58	4.96 ± 1.52

Source: Author

63.73BF + 84.77CD - 86.29 CE + 19.13 CF + 27.86 DE - 26.35DF + 33.85EF - 47.75A^2 - 166.77B^2 - 150.69C^2 + 87.44D^2 + 50.19E^2 - 18.99F^2

where Y is the concentration of cordycepin (g/L), A is the concentration of glucose (g/L), B is the concentration of adenine (g/L), C is the concentration of glycine (g/L), D is the concentration of alanine (g/L), E is the concentration of casein hydrolysate (g/L), and F is the concentration of vitamin solution (mL/L).

According to the model using CCD, the optimal medium for cordycepin production by *C. militaris* BCC 2819 consisted of glucose 60 g/L, adenine 4.41 g/L, glycine 6.4 g/L, alanine 10 g/L, glutamine 2 g/L, casein hydrolysate 5 g/L, peptone 10 g/L, yeast extract 15 g/L, $(NH_4)_2SO_4$ 2 g/L, $MgSO_4$ 0.5 g/L, KH_2PO_4 0.5 g/L, K_2HPO_4 0.5 g/L, trace element 2 mL/L and vitamin solution 4 mL/L.

The cordycepin production model of *C. cicadae* BCC 19788 is shown in Table S4. The *p* value (p = 0.0245) for the model and for the lack of fit (p = 0.74) demonstrated that the experimental data fit well with the model. The model showed a determination coefficient value (R^2) of 0.78 for cordycepin production, indicating that the model could explain up to 78% of the observed variation in the response.

The equation that correlated the six factors and cordycepin production level by *C. cicadae* BCC 19788 was as follows:

Y = 94.2120 - 16.494A - 40.175B + 46.319C - 39.10D + 448.46E + 70.16F + 240.50AB + 187.57AC + 516.02AD - 317.20AE + 37.71AF + 107.16BC + 6.21BD - 203.31BE + 636.80BF - 277.48CD + 178.76CE -457.05CF - 230.83DE + 314.59DF + 29.42EF + 163.67A^2 - 384.31B^2 + 134.30C^2 + 159.93D^2 + 185.92E^2 + 26.17F^2

where Y is the concentration of cordycepin (g/L), A is the concentration of $(NH_4)_2SO_4$ (g/L), B is the concentration of adenine (g/L), C is the concentration of glycine (g/L), D is the concentration of alanine (g/L), E is the concentration of casein hydrolysate (g/L), and F is the concentration of vitamin solution (mL/L).

According to the model using CCD, the optimal medium for cordycepin production by *C. cicadae* BCC 19788 consists of glucose 20 g/L, adenine 5 g/L, glycine 30 g/L, alanine 5 g/L, glutamine 2 g/L, casein hydrolysate 10 g/L, peptone 10 g/L, yeast extract 15 g/L, $(NH_4)_2SO_4$ 5.68 g/L, $MgSO_4$ 0.5 g/L, KH_2PO_4 0.5 g/L, K_2HPO_4 0.5 g/L, trace element 2 mL/L and vitamin solution 6 mL/L.

Cordycepin production by *C. militaris* BCC 2819 and *C. cicadae* BCC 19788 in batch fermenters

To evaluate the optimal medium for cordycepin production obtained by CCD experiment, batch fermentation was performed with optimal medium determined from previous experiments at a working volume of 3 L in a 5-L fermenter. The highest cordycepin production of 2,598.44 ± 57.16 and 2,998.44 ±20.11 mg/L was produced by *C. militaris* BCC 2819 and *C. cicadae* BCC 19788 at 120 and 72 h, respectively, as shown in Figure 2A and B.

For other bioactive compounds, *C. militaris* BCC 2819 *and C. cicadae* BCC 19788 produced the highest adenosine and mannitol contents of 3,158.58 ± 287.45 and 4,580.35 ± 287.14 mg/L, respectively. Moreover, the highest cell mass and exopolysaccharide content produced by *C. militaris* BCC 2819 were 56.08 ± 1.29 and 42.18 ± 1.40 g/L, respectively (Figure 1A). The highest cell mass and exopolysaccharide content produced by *C. cicadae* BCC 19788 were 45.23 ± 3.41 and 44.08 ± 3.55 g/L, respectively (Figure 1B). This result demonstrated that the optimal medium from the CCD experiment could be used at a larger production scale of 5-L fermenter.

Cordycepin production by *C. militaris* BCC 2819 and *C. cicadae* BCC 19788 in fed-batch fermenters with an exponential feed rate

In this experiment, fed-batch fermentation was performed to increase the concentration of cordycepin by both fungal strains. The highest cordycepin production by *C. militaris* BCC 2819 and *C. cicadae* BCC 19788 reached 3,112.50 ± 712.00 and 3,587.10 ± 247.70 mg/L, respectively, as shown in Figure 4. For the other bioactive compounds (adenosine and mannitol), *C. militaris* BCC 2819 produced 2,897.40 ± 382.47 and 5,981.10 ± 254.72 mg/L, and *C. cicadae* BCC 19788 produced 3,78520 ± 165.70 and 6,100.20 ± 191.14 mg/L, respectively, as shown in Figure 4A and B. Furthermore, the highest cell mass and exopolysaccharide content of *C. militaris* BCC 2819 reached 66.35 ± 1.91 and 43.90 ± 2.51 g/L, while those of *C. cicadae* BCC 19788 reached 63.81 ± 1.03 and 38.10 ± 2.84 g/L, respectively, as shown in Figure 3A and B. This result indicated that fed-batch fermentation could be used to improve the production in term of concentration of cordycepin and the other bioactive compounds by *C. militaris* BCC 2819 and *C. cicadae* BCC 19788.

DISCUSSION

In this study, the two potential candidate strains of *C. militaris* BCC 2819 and *C. cicadae* BCC 19788 were selected to develop cordycepin production processes in submerged fermentation based on our prelimary screening data (data not shown). The media compositions were screened and selected using statistical experiments to favor and improve higher production of cordycepin.

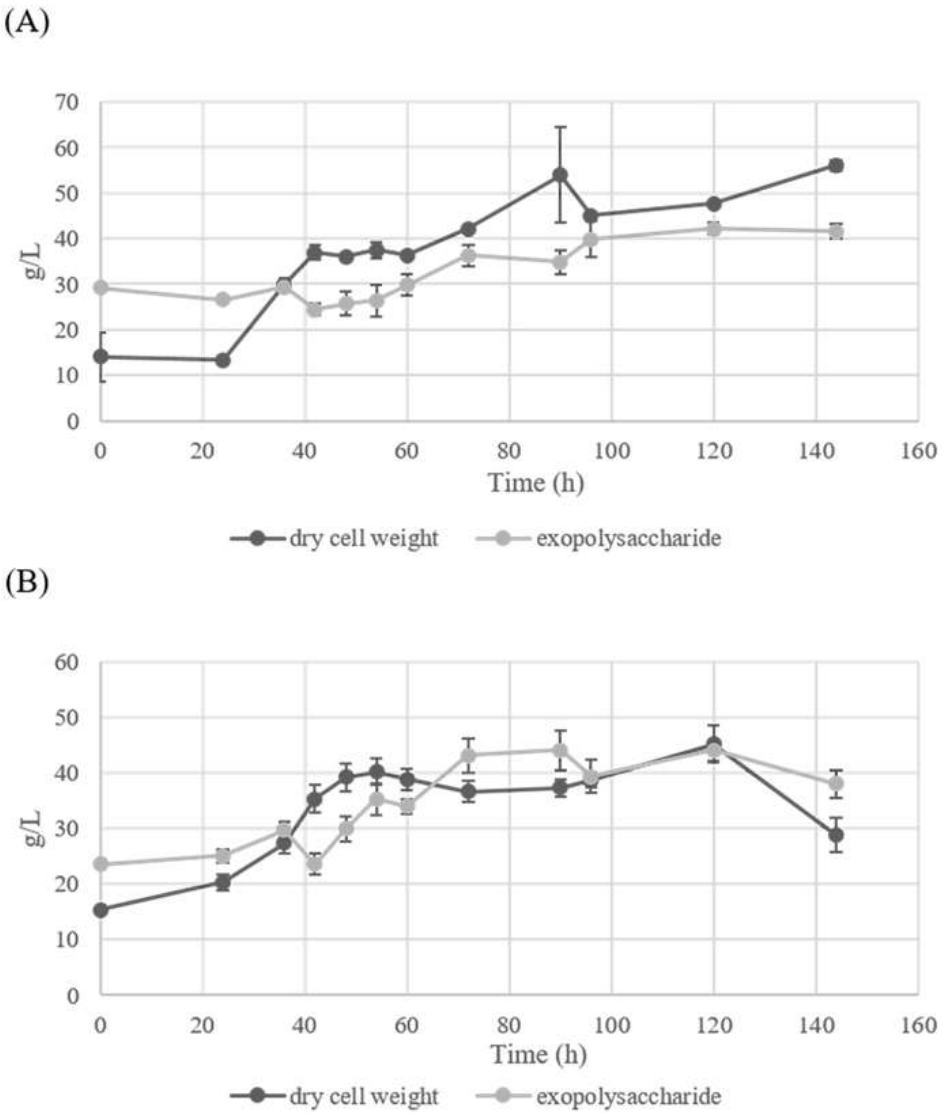

Figure 1. Dry cell weight and exopolysaccharide content of *C .militaris* BCC 2819 (A) and *C. cicadae* BCC 19788 (B) cultivated in batch fermentation.
Source: Author

The results showed that glucose, adenine, glycine, alanine, casein hydrolysate and vitamin solution favored the cordycepin production of *C. militaris* BCC 2819, while $(NH_4)_2SO_4$ affected the cordycepin production of *C. cicadae* BCC 19788 instead of glucose. These results were similar to the report of Mao et al. (2005) in which they demonstrated that glucose was the most suitable carbon source for cordycepin production of *C. militaris* (Mao et al., 2005) and Lee et al. (2019) reported that casein hydrolysate was the most beneficial nitrogen source for cordycepin production of *C. militaris* KYL05. Das et al. (2010) and Mao et al. (2005) demonstrated the improvement of cordycepin production of *C. militaris* using yeast extract and peptone in the medium compositions and obviously improved cordycepin production. Moreover, glycine, glutamine and alanine were reported as additives for the improvement of cordycepin production (Das et al., 2009; Wen et al., 2016). Furthermore, adenosine was also used as an additive for cordycepin production as shown in Vikas et al. (2020) report.

In this study, a significant improvement in cordycepin production was achieved by formulating the optimal medium for submerged fermentation. The highest cordycepin production by *C. militaris* BCC 2819 and *C. cicadae* BCC 19788 reached 3,112.50 and 3,587.10 mg/L

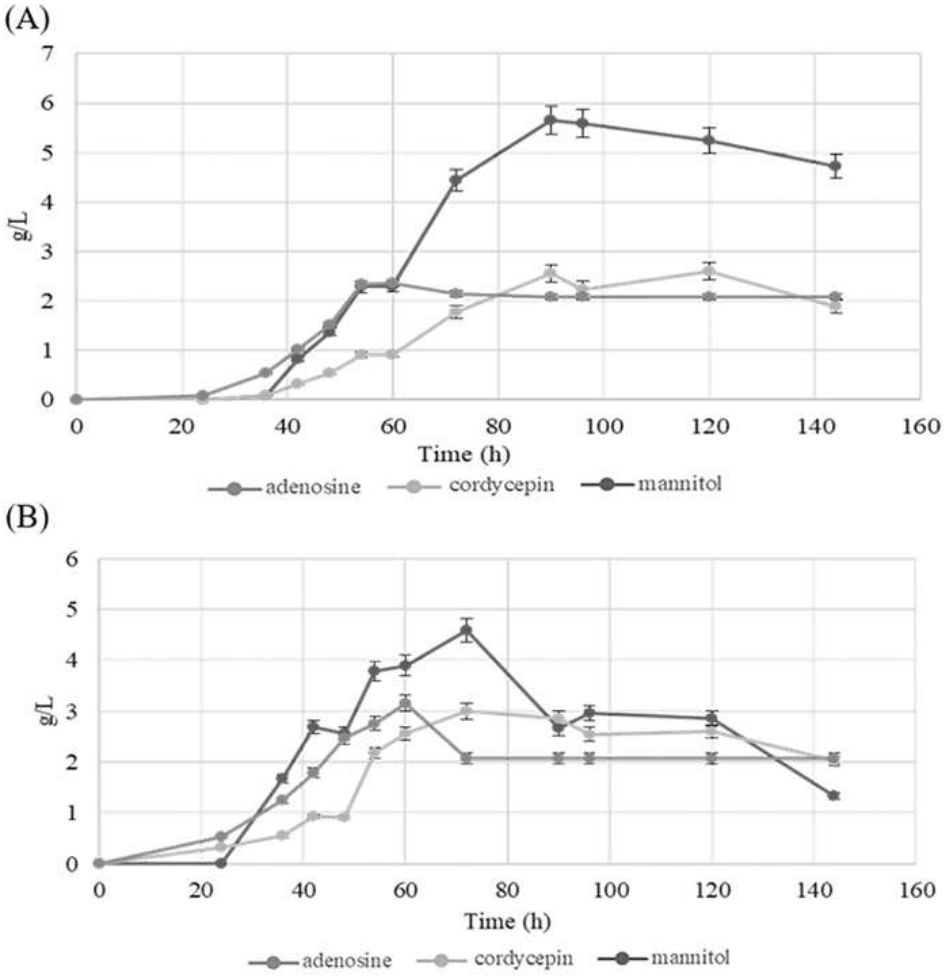

Figure 2. Adenosine, cordycepin and mannitol production by *C .militaris* BCC 2819 (A) and *C. cicadae* BCC 19788 (B) cultivated in batch fermentation
Source: Author

in the 5-L fed-batch fermenter, respectively. Compared to other studies, Das et al. (2010) reported that a mutant *C. militaris* strain generated by ion beam irradiation produced 6,840 mg/L cordycepin in a 100-mL culture. Tang et al. (2018) reported a production of 5,290 mg/L cordycepin via two-step culture of *C. militaris* in a 100-mL culture. However, on a larger scale, Mao and Zhong (2004) reported that *C. militaris* produced 201.1 mg/L cordycepin by using two-stage dissolved oxygen control in a 5-L fermenter. Moreover, Mao and Zhong (2006) reported the production of 346.1 mg/L cordycepin in a 3.5-L fermenter through fed-batch fermentation with $NH4^+$ feeding. In a previous report, the yield of cordycepin decreased in the scale up step to the fermenter. In our study, the cordycepin yield slightly decreased after performing the validation in the fermenter. The present study reported the highest cordycepin production in fermenters by submerged fermentation using the new isolates *C. militaris* BCC 2819 and *C. cicadae* BCC 19788 as fugal cell factories.

In addition to cordycepin, additional bioactive compounds, including adenosine, mannitol and exopolysaccharide, were produced by *Cordyceps*. The pharmacological effects of adenosine have been reported. Adenosine can be used as a cardioprotective and therapeutic agent for chronic heart failure (Kitakaze and Holi, 2000), and it could also inhibit the release of neurotransmitters in the central nervous system (Ribeiro, 1995). Polysaccharides are considered to possess anti-inflammatory, antioxidant (Wen et al., 2013), antitumor (Zhang et al., 2007), antimetastatic, immunomodulatory, hypoglycemic activity (Kiho et al., 1996), steroidogenic, and hyperlipidemia. Mannitol (cordycepic acid) has diuretic, anti-tussive and anti-free

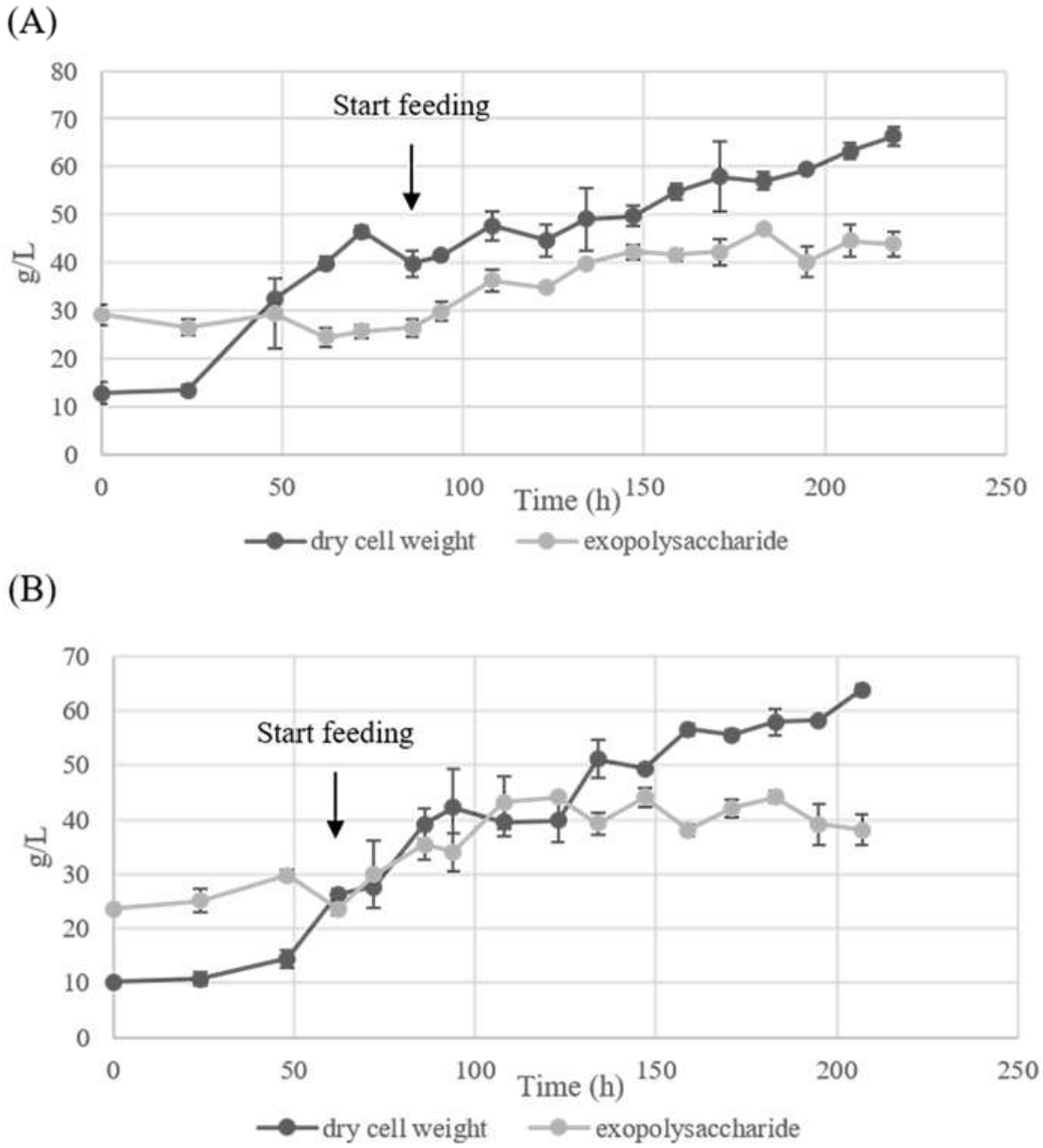

Figure 3. Dry cell weight and exopolysaccharide content of *C .militaris* BCC 2819 (A) and *C. cicadae* BCC 19788 (B) cultivated in fed-batch fermentation.
Source: Author

radical activities (Li et al., 2006). In this study, the highest yield of cordycepin was produced, and both fungal strains also produced adenosine, mannitol and exopolysaccharide at high yield in optimal medium with fermentation processes. These results demonstrated the optimal cordycepin production media, fermentation processes and cordycepin-producing strains with high potential for applications in cordycepin production on pilot and industrial scales.

Conclusion

The present study successfully optimized medium for cordycepin production, a potential bioactive compound in cordyceps, along with the production of other bioactive compounds of *C. militaris* BCC 2819 and *C. cicadae* BCC 19788. These two fungal cell factories are new high cordycepin-producing fungal isolates that were isolated from natural resources. Compared with the unoptimized

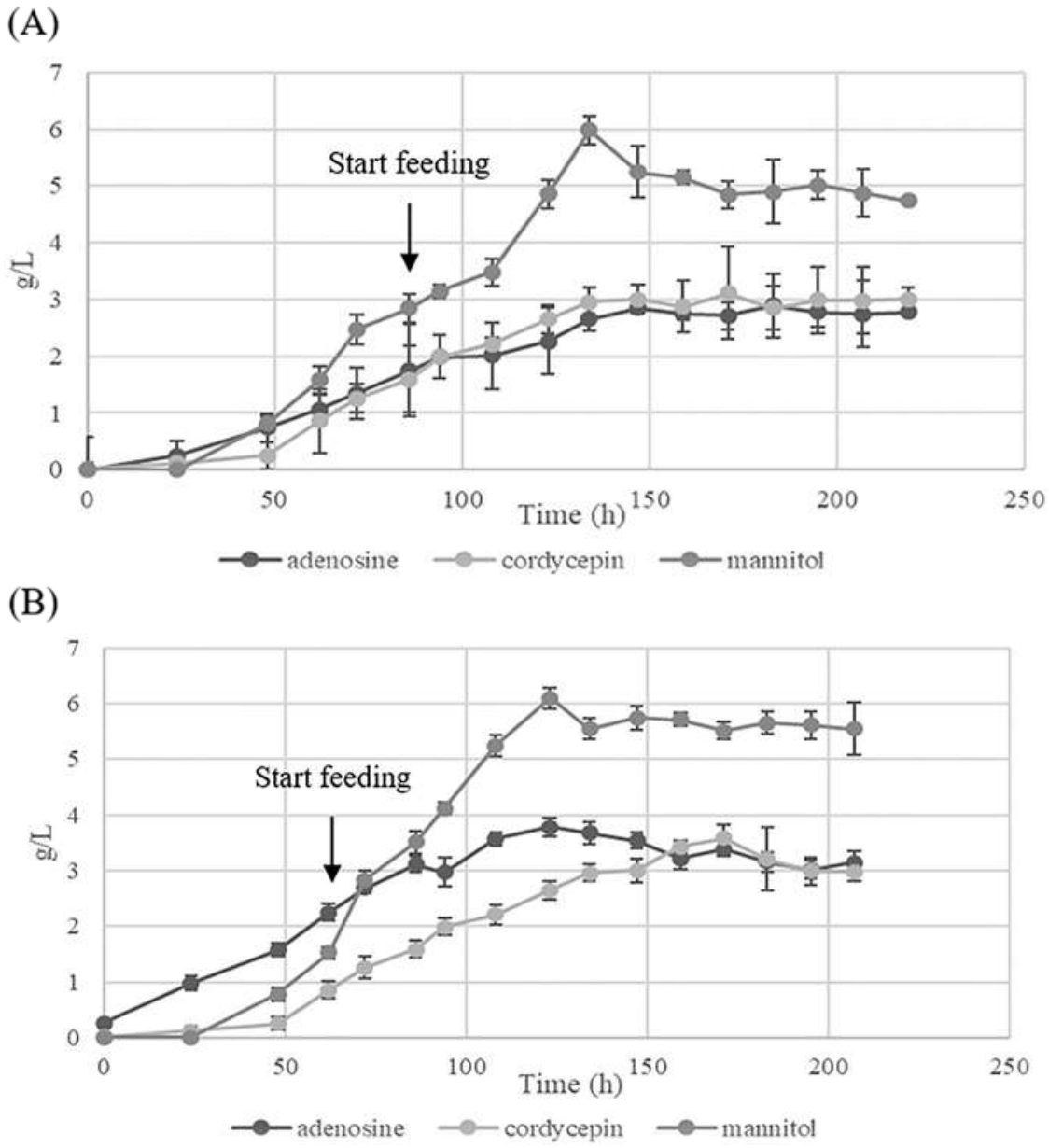

Figure 4. Adenosine, cordycepin and mannitol production by *C .militaris* BCC 2819 (A) and *C. cicadae* BCC 19788 (B) cultivated in fed-batch fermentation.
Source: Author

conditions, cordycepin production in fed-batch fermentation increased up to 45-fold in *C. militaris* BCC 2819 and up to 90-fold in *C. cicadae* BCC 19788. Moreover, other bioactive compounds, including adenosine, mannitol and exopolysaccharide, were also produced at high yields. This result showed that the optimized medium and processes developed for the production of cordycepin and other bioactive compounds by *C. militaris* BCC 2819 and *C. cicadae* BCC 19788 will be new approaches for commercial-scale production that replace the conventional process of solid-state fermentation. This process can also be used on an industrial scale, potentially with a shortened cultivation period for higher productivity and lower production costs.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY

Table S1. ANOVA of cordycepin production by *C .militaris* BCC 2819 using the Plackett-Burman design.

Source	Sum ofsquares	df	Meansquare	FValue	p-value (Prob > F)	
Block	0.16	2	0.079			
Model	2.86	24	0.12	3.30	0.0021	significant
A-Glucose	2.06	1	2.06	57.02	< 0.0001	
B-Peptone	0.026	1	0.026	0.72	0.4054	
C-Yeast extract	0.078	1	0.078	2.14	0.1557	
D-$(NH_4)_2SO_4$	0.032	1	0.032	0.89	0.3543	
E-Adenine	0.048	1	0.048	1.34	0.2587	
F-Glutamine	0.023	1	0.023	0.65	0.4291	
G-Glycine	0.098	1	0.098	2.71	0.1122	
H-Alanine	0.045	1	0.045	1.26	0.2730	
J-Casein hydrolysate	0.19	1	0.19	5.13	0.0324	
K-Vitamin solution	0.049	1	0.049	1.35	0.2563	
L-Trace element	0.026	1	0.026	0.71	0.4061	
AB	0.022	1	0.022	0.61	0.4430	
AC	0.035	1	0.035	0.96	0.3375	
AD	0.033	1	0.033	0.90	0.3507	
AE	0.051	1	0.051	1.42	0.2451	
AF	0.054	1	0.054	1.48	0.2348	
AG	0.039	1	0.039	1.09	0.3069	
AH	0.087	1	0.087	2.41	0.1332	
AJ	0.25	1	0.25	7.00	0.0139	
AK	0.081	1	0.081	2.24	0.1468	
AL	0.051	1	0.051	1.42	0.2441	
BC	0.028	1	0.028	0.79	0.3836	
ABC	0.063	1	0.063	1.74	0.1988	
ABCD	0.074	1	0.074	2.04	0.1651	
Residual	0.90	25	0.036			
Lack of Fit	0.89	24	0.037	3.52	0.4013	not significant
Pure Error	0.011	1	0.011			
Cor Total	3.92	51				
R-Squared	0.76					

Source: Author

Table S2. ANOVA of cordycepin production by *I .cicadae BCC 19788* using the Plackett-Burman design.

Source	Sum of squares	df	Mean square	FValue	p-Value (Prob > F)	
Block	3.39	2	1.70			
Model	31.82	24	1.33	2.45	0.0150	Significant
A-Glucose	3.75	1	3.75	6.94	0.0143	
B-Peptone	0.56	1	0.56	1.03	0.3198	
C-Yeast extract	0.65	1	0.65	1.20	0.2835	
D-$(NH_4)_2SO_4$	0.79	1	0.79	1.46	0.2383	
E-Adenine	0.63	1	0.63	1.17	0.2896	
F-Glutamine	0.21	1	0.21	0.40	0.5347	
G-Glycine	0.45	1	0.45	0.84	0.3691	
H-Alanine	2.50	1	2.50	4.61	0.0416	
J-Casein hydrolysate	0.43	1	0.43	0.80	0.3787	
K-Vitamin solution	0.72	1	0.72	1.33	0.2589	
L-Trace element	1.70	1	1.70	3.14	0.0887	
AB	8.11	1	8.11	14.99	0.0007	
AC	0.21	1	0.21	0.39	0.5400	
AD	0.94	1	0.94	1.74	0.1986	
AE	0.87	1	0.87	1.61	0.2164	
AF	0.79	1	0.79	1.46	0.2379	
AG	0.55	1	0.55	1.02	0.3216	
AH	0.68	1	0.68	1.26	0.2726	
AJ	0.021	1	0.021	0.040	0.8438	
AK	6.577E-003	1	6.577E-003	0.012	0.9131	
AL	1.01	1	1.01	1.86	0.1844	
BC	0.93	1	0.93	1.72	0.2011	
ABC	0.92	1	0.92	1.70	0.2037	
ABCD	1.07	1	1.07	1.97	0.1725	
Residual	13.53	25	0.54			
Lack of Fit	13.13	24	0.55	1.38	0.5975	Not significant
Pure Error	0.40	1	0.40			
Cor Total	48.74	51				
R-Squared	0.70					

Source: Author

Table S3. ANOVA of cordycepin production by *C .militaris* BCC 2819 using the central composite design.

Source	Sum of squares	df	Mean square	FValue	p-Value (Prob > F)	
Model	2.793E+006	27	1.035E+005	11.43	< 0.0001	significant
A-Glucose	1.374E+005	1	1.374E+005	15.18	0.0010	
B-Adenine	9961.64	1	9961.64	1.10	0.3074	
C-Glycine	4403.04	1	4403.04	0.49	0.4940	
D-Alanine	33218.73	1	33218.73	3.67	0.0706	
E-Casein	82156.05	1	82156.05	9.07	0.0072	
F-Vitamin solution	5249.98	1	5249.98	0.58	0.4557	
AB	34638.50	1	34638.50	3.83	0.0653	
AC	4550.85	1	4550.85	0.50	0.4870	
AD	26422.47	1	26422.47	2.92	0.1039	
AE	4.07	1	4.07	4.490E-004	0.9833	
AF	29445.34	1	29445.34	3.25	0.0872	
BC	1763.84	1	1763.84	0.19	0.6639	
BD	1.425E+005	1	1.425E+005	15.74	0.0008	
BE	84730.73	1	84730.73	9.36	0.0065	
BF	17264.07	1	17264.07	1.91	0.1834	
CD	2.300E+005	1	2.300E+005	25.40	< 0.0001	
CE	2.383E+005	1	2.383E+005	26.32	< 0.0001	
CF	1554.97	1	1554.97	0.17	0.6832	
DE	3298.20	1	3298.20	0.36	0.5533	
DF	22209.97	1	22209.97	2.45	0.1338	
EF	36676.00	1	36676.00	4.05	0.0585	
A^2	30418.42	1	30418.42	3.36	0.0825	
B^2	1.819E+005	1	1.819E+005	20.09	0.0003	
C^2	3.029E+005	1	3.029E+005	33.46	< 0.0001	
D^2	1.020E+005	1	1.020E+005	11.26	0.0033	
E^2	33597.28	1	33597.28	3.71	0.0692	
F^2	4812.06	1	4812.06	0.53	0.4749	
Residual	1.720E+005	19	9054.35			
Lack of Fit	14596.77	1	14596.77	1.67	0.2127	not significant
Pure Error	1.574E+005	18	8746.44			
Cor Total	2.966E+006	46				
R-Squared	0.94					

Source: Author

Table S4. ANOVA of cordycepin production by *I .cicadae* BCC 19788 using the central composite design.

Source	Sum of squares	df	Mean square	FValue	p-Value (Prob > F)	
Model	2.782E+007	27	1.031E+006	2.43	0.0245	significant
A)-$NH_4(_2SO_4$	1.196E+006	1	1.196E+006	2.82	0.1095	
B-Adenine	1.507E+005	1	1.507E+005	0.36	0.5582	
C-Glycine	4.999E+005	1	4.999E+005	1.18	0.2913	
D-Alanine	7489.24	1	7489.24	0.018	0.8957	
E-Casein hydrolysate	9.853E+005	1	9.853E+005	2.32	0.1440	
F-Vitamin solution	24114.78	1	24114.78	0.057	0.8141	
AB	1.851E+006	1	1.851E+006	4.36	0.0504	
AC	1.126E+006	1	1.126E+006	2.65	0.1198	
AD	1.131E+006	1	1.131E+006	2.67	0.1189	
AE	4.275E+005	1	4.275E+005	1.01	0.3281	
AF	45512.76	1	45512.76	0.11	0.7468	
BC	48786.51	1	48786.51	0.11	0.7383	
BD	1235.61	1	1235.61	2.912E-003	0.9575	
BE	1.323E+006	1	1.323E+006	3.12	0.0935	
BF	1.723E+006	1	1.723E+006	4.06	0.0583	
CD	2.464E+006	1	2.464E+006	5.81	0.0263	
CE	1.023E+006	1	1.023E+006	2.41	0.1370	
CF	8.875E+005	1	8.875E+005	2.09	0.1644	
DE	2.264E+005	1	2.264E+005	0.53	0.4740	
DF	3.167E+006	1	3.167E+006	7.46	0.0132	
EF	27698.15	1	27698.15	0.065	0.8011	
A^2	3.573E+005	1	3.573E+005	0.84	0.3703	
B^2	1.970E+006	1	1.970E+006	4.64	0.0442	
C^2	2.406E+005	1	2.406E+005	0.57	0.4606	
D^2	3.412E+005	1	3.412E+005	0.80	0.3811	
E^2	4.611E+005	1	4.611E+005	1.09	0.3103	
F^2	9136.11	1	9136.11	0.022	0.8849	
Residual	8.061E+006	19	4.243E+005			
Lack of Fit	51211.00	1	51211.00	0.12	0.7384	not significant
Pure Error	8.010E+006	18	4.450E+005			
Cor Total	3.588E+007	46				
R-Squared	0.78					

Source: Author