Effect of Temperature on Cordycepin Production in *Cordyceps militaris*

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Abstract

Fifteen *Cordyceps militaris* strains were evaluated for the production of cordycepin (3'-deoxyadenosine) by static culture in Erlenmeyer flasks. Cordycepin production was shown to be strain dependent with the highest yields of 544.82 ± 99.80 mg L^{-1} (36.32 ± 6.65 mg L^{-1} d^{-1}) and 587.68 ± 16.82 mg L^{-1} (39.18 ± 1.12 mg L^{-1} d^{-1}) from *C. militaris* BCC2816 and BCC2819, respectively, at 25°C. Other *C. militaris* strains produced significantly lower cordycepin yields. The effect of temperature on mycelial growth and cordycepin production was studied. Optimum temperature for mycelial growth and cordycepin production was 15 to 20°C and 25°C, respectively, and at 30°C, both mycelial growth and cordycepin production ceased.

Keywords: entomopathogenic fungi, fermentation, insect fungi, liquid culture

Introduction

Cordyceps militaris (L.) Link (Clavicipitaceae) is an entomopathogenic fungus, which infects and grows parasitically on Lepidoptera larvae in temperate countries (Isaka et al., 2005). Traditionally, this fungus has been used as a nutraceutical and in traditional Chinese medicine, particularly for patients suffering from cancer (Ng and Wang, 2005). Cordycepin (3'-deoxyadenosine) is the main bioactive metabolite of *C. militaris*. Other useful bioactivities include: anti-viral, anti-fungal, anti-bacterial, anti-leukemic activities, and anti-metastatic action on some cancer cell lines (Koc et al., 1996; Ahn et al., 2000; Nakamura et al., 2006). Cordycepin was first extracted from *C. militaris* (Cunningham et al., 1950), and then found to be produced by *C. sinensis* and *C. kyushuensis* (Ling et al., 2002). Although cordycepin can be chemically synthesised (McDonald et al., 1996; Hansske et al., 1985), yields are low and the processes are complicated. The natural fruiting bodies of *Cordyceps* are very rare and costly to collect. Fruiting body production *in vitro* is not repeatable and cordycepin content of natural *Cordyceps* is much lower than that of cultured mycelia (Guo et al., 1998). Cultivation of *C. militaris* mycelium using artificial media has recently been developed in which several methods have been reported for cordycepin production such as surface culture (Masuda et al., 2007) and submerged culture (Mao et al., 2005). The first method (Masuda et al., 2007) gave higher cordycepin yields. However, only a single *C. militaris* strain was employed and cordycepin production may vary with different strains.

Although submerged cultivation of *C. militaris* for efficient production of valuable metabolites has been studied extensively, only the effect of initial pH value, mode of propagation and carbon and nitrogen sources were reported (Shih et al., 2007). It is a well-known fact that the growth rate and

metabolite production of fungus is affect by many conditions, with the incubation temperature being an important one. As far as we know, there is limited knowledge about the temperature effect for cordycepin production by *C. militaris*, and there have been no reports on temperature optimization to improve cordycepin production. In this work, the effects of temperature on mycelial growth and cordycepin production were focused in order to improve the cordycepin production of *C. militaris* strains. It could be the evidence whether cordycepin is atemperature-related/dependent metabolite since the reported producers so far known by us are cold-temperate fungi. The information obtained is considered fundamental and useful to the development of *C. militaris* cultivation process for efficient production of cordycepin on a large scale.

Materials and Methods

Microorganisms and Seed Cultures

Cordyceps militaris strains were obtained from BIOTEC Culture Collection (BCC), National Center for Genetic Engineering and Biotechnology, Bangkok, Thailand and NITE Biological Resource Center (NBRC), National Institute of Technology and Evaluation, Chiba, Japan (Table 1). All cultures were maintained in cryotubes at -80°C in 10% glycerol. Cultures were activated in potato dextrose agar (PDA) for seven days. To prepare seed cultures, mycelia on PDA were cut into small pieces (~1x1 mm^2 per piece) and transferred into 250 mL Erlenmeyer flasks containing 100 mL Difco™ potato dextrose broth (16 pieces per flask). Seed cultures were incubated in a rotary shaker at 150 rpm, at 25°C for 7 days.

Fermentation

The fermentation medium, was modified from Mao et al. (2005), (glucose (40 g L^{-1}), peptone (15 g L^{-1}), KH_2PO_4 (0.5 g L^{-1}), K_2HPO_4 (0.5 g L^{-1}) and $MgSO_4.7H_2O$ (0.5 g L^{-1}). Experiments were conducted in 250 mL Erlenmeyer flasks containing 45 mL medium. Flasks were inoculated with 5 mL of seed culture and then incubated at four different temperatures (15, 20, 25 and 30°C) in the dark and static conditions for 15 days.

Determination of Mycelial Dry Weight (DW)

Cultures were filtrated through pre-weighed filter papers (Whatman, Gemany) in order to separate culture broth and mycelium, then dried at 65°C and weighed. The filtrate was stored at -20°C in a freezer for later determination of cordycepin yield.

Determination of Cordycepin Production

Two mg of cordycepin (Fluka BioChemika AG, Germany) was dissolved in 2 mL of pure water and diluted at various concentrations for standard calibration. Culture filtrates were filtered through cellulose acetate filter paper (0.2 µm pore size) and cordycepin concentration was determined by using High Performance Liquid Chromatography (HPLC).

Table 1 List and detail of 15 *C. militaris* cultures.

Fungal strain	Source of collection	Collection date
C. militaris BCC1974	Data not available	Data not available
C. militaris BCC1975	Data not available	1998
C. militaris BCC2790	Nyutu Spa, Lake Tazawa, Tazawako Town, Japan	July 19, 2000
C. militaris BCC2814	Tsuchiyu Hot Spring, Fukushima City, Japan	July 21, 2000
C. militaris BCC2815	Appi Highlands, Ahiro Town, Japan	July 18, 2000
C. militaris BCC2816	Appi Highlands, Ahiro Town, Japan	July 18, 2000
C. militaris BCC2817	Appi Highlands, Ahiro Town, Japan	July 18, 2000
C. militaris BCC2818	Appi Highlands, Ahiro Town, Japan	July 18, 2000
C. militaris BCC2819	Appi Highlands, Ahiro Town, Japan	July 18, 2000
C. militaris BCC2824	Nyutu Spa, Lake Tazawa, Tazawako Town, Japan	July 19, 2000
C. militaris BCC2826	Nyutu Spa, Lake Tazawa, Tazawako Town, Japan	July 19, 2000
C. militaris BCC2838	Nyutu Spa, Lake Tazawa, Tazawako Town, Japan	July 19, 2000
C. militaris NBRC5298	Data not available	Data not available
C. militaris NBRC9787	Ohdaigaharayama, Nara Pref., Japan	Data not available
C. militaris NBRC100741	Hioki, Miyazu, Kyoto, Japan	Data not available

A C_{18} column (Phenonemex, USA) with 4.6x200 mm and 4 µm particle size was used for sample separation. The mobile phase consisted of 10 mM KH_2PO_4 dissolved in the mixture of water and methanol (85:15) (Mao et al., 2005) and driven by a Waters 626 double pump at 1 mL min^{-1}. The process was controlled by a Waters 600S controller. The UV wavelength was monitored at 254 nm using a Waters 996 Photodiode Array Detector (Millipore, USA). Fifty µL samples were injected using a micro syringe.

Data Analysis

Dry weight and cordycepin yield are expressed as means ± SD. An Analysis of Variance (ANOVA) followed by Tukey's test was applied for multiple comparisons of significant analyses at $P<0.05$. Statistical data analyses were performed in SPSS version 13.0 software packet.

Results

Mycelial Growth

After fifteen-day incubation, biomass and liquid culture at four temperature conditions were separated, and then mycelial biomass weight was measured in dry. Table 2 summarises the mycelial dry weights of 15 *C. militaris* strains at four temperatures. The highest biomass was observed in *C. militaris* BCC2790 grown at 15°C (16.59 ± 0.29 g L^{-1}) and *C. Militaris* NBRC5298, and NBRC9787 also showed high biomass at this temperature. Most *C. militaris* strains (BCC1974, BCC1975, BCC2816, BCC2817, BCC2818, BCC2819 and BCC2826) produced high biomass at 20°C. Dry weight of the other *C. militaris* strains (BCC2814, BCC2815, BCC2824, BCC2838, and NBRC100741) were not temperature significant. The biomass weights of all strains were very low at 30°C. In the observation, mycelial growth of all 15 *C. militaris* strains was similar. It was white, dense and completely covered the surface of the culture broth at 15, 20 and 25°C, but at 30°C it was rare and submerged in the medium (Figure 1). Therefore, it can be mentioned that growth of different strains was not resembled each other in the changes of temperature and high temperature caused a growth inhibition of this fungus.

Cordycepin Production

Cordycepin yields and productivities of *C. militaris* strains are shown in Table 3 and Figure 2. Significant differences in cordycepin production were found between the different strains with only a trace amount in *C. militaris* NBRC5298. The highest yields at 25°C, was by *C. militaris* strains BCC2816 and BCC2819 of 544.82 ± 99.80 mg L^{-1} and 587.68 ± 16.82 mg L^{-1}, and correlative productivities of 36.32 ± 6.65 mg L^{-1} d^{-1} and 39.18 ± 1.12 mg L^{-1} d^{-1}, respectively. Significant lower cordycepin production was noted for the other strains.

Discussions

Previous studies of cordycepin production from *C. militaris* have focused on optimizing growth conditions and media. Only one strain was used in these studies, namely *C. militaris* NBRC9787 which gave a yield of up to 2.5 g L^{-1} on an optimized nutrient source and fermentation conditions (Masuda et al., 2007). However in the current study this strain produced only 104.46 ± 45.45 mg L^{-1} cordycepin. It could be the effect of the medium in fermentation. The current study has focused on the evaluation of strains to maximize cordycepin production. Variation was observed between the different strains with the highest yields from *C. militaris* BCC2816 and BCC2819, which produced a five-fold higher amount of cordycepin than the previously screened strain *C. militaris* NBRC9787 at 25°C (Masuda et al., 2007). In addition, other study on *C. militaris* BCC2816

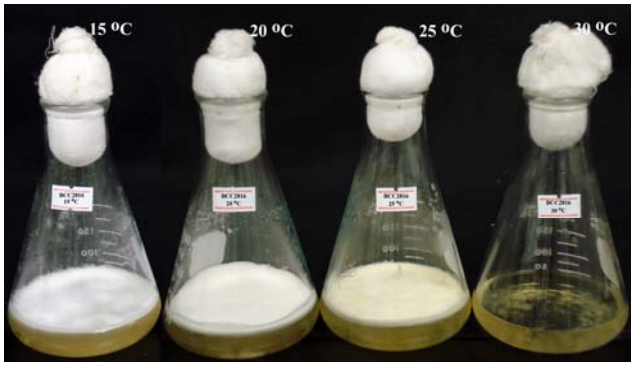

Figure 1 Mycelium of *C. militaris* BCC2816 grew on the medium surface after 15 days cultivation in different temperatures.

Table 2 Mycelial dry weight of 15 C. militaris strains at four cultivation temperatures after 15 days cultivation[1/].

Fungal strain	Mycelial dry weight (g L^{-1})			
	15 °C	20 °C	25 °C	30 °C
C. militaris BCC1974	8.71 ± 2.14ab	9.53 ± 0.47b	7.82 ± 0.86ab	3.80 ± 0.25a
C. militaris BCC1975	11.56 ± 2.05b	11.16 ± 0.52b	9.69 ± 0.63b	3.75 ± 0.27a
C. militaris BCC2790	16.59 ± 0.29b	7.28 ± 2.53a	6.66 ± 0.13a	5.06 ± 0.66a
C. militaris BCC2814	8.69 ± 1.27a	9.47 ± 3.11a	6.46 ± 1.02a	3.48 ± 0.48a
C. militaris BCC2815	5.25 ± 0.26a	6.33 ± 0.60a	4.96 ± 1.12a	3.63 ± 0.97a
C. militaris BCC2816	3.88 ± 1.69a	11.27 ± 1.90b	8.40 ± 1.01ab	4.46 ± 0.78a
C. militaris BCC2817	6.51 ± 1.64ab	9.19 ± 0.55b	7.81 ± 0.20ab	4.18 ± 1.12a
C. militaris BCC2818	3.63 ± 0.55a	7.70 ± 0.54c	6.50 ± 1.06bc	4.72 ± 0.11ab
C. militaris BCC2819	4.99 ± 1.78a	10.37 ± 0.17b	10.19 ± 0.16b	3.50 ± 0.41a
C. militaris BCC2824	5.42 ± 0.98a	6.60 ± 0.04a	6.72 ± 0.27a	4.88 ± 0.50a
C. militaris BCC2826	8.90 ± 0.06bc	10.36 ± 1.39c	6.47 ± 0.06ab	4.02 ± 0.51a
C. militaris BCC2838	6.25 ± 1.03a	5.43 ± 1.25a	4.88 ± 0.23a	3.15 ± 0.69a
C. militaris NBRC5298	9.20 ± 0.94b	7.50 ± 1.50ab	4.74 ± 0.36a	5.48 ± 0.52ab
C. militaris NBRC9787	13.53 ± 3.92b	7.08 ± 0.06ab	6.63 ± 1.77ab	4.61 ± 0.09a
C. militaris NBRC100741	7.55 ± 0.87a	6.67 ± 0.26a	5.32 ± 0.62a	4.09 ± 1.32a

[1/] All values are means of duplicate experiments ± SD. Values with same letters within a row are not significantly different at P<0.05.

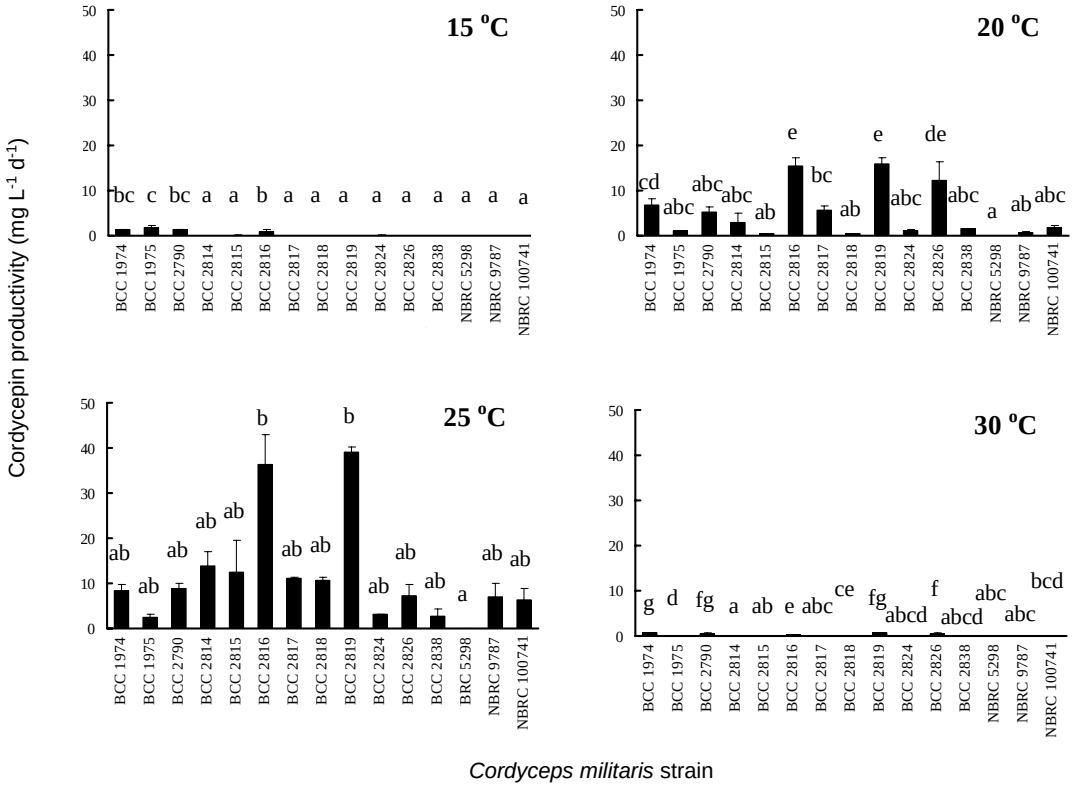

Figure 2 Comparison of cordycepin productivity (mg L^{-1} d^{-1}) of 15 *C. militaris* strains in four cultivation temperatures, A: 15 °C; B: 20 °C; C: 25 °C and D: 30 °C. Error bars show SD of means of duplicate experiments. Values with same letters within a chart are not significantly different at P<0.05.

reported that *C. militaris* BCC2816 produced 36 mg in total 5 litters of potato dextrose broth medium at 25°C (Rukachaisirikul et al., 2004). The current study, this strain yields up to 544.82 ± 99.80 mg L^{-1} in a different culture medium. It indicates that medium composition and fermentation conditions are important factors for cordycepin production. *C. militaris* BCC2816 and BCC2819 were the highest producers of cordycepin in this study and should be key taxa for further optimization studies.

The fermentation temperature at 25°C was used for cordycepin and other nucleosides production in previous studies (Shih et al., 2007; Masuda et al., 2007; Mao et al., 2005). The current study showed that the low temperature at 15 to 20°C was not favorable for cordycepin production in most strains even though mycelium developed well in the temperatures ranged from 15 to 25°C in static liquid culture of *C. militaris*. The temperature at 30°C caused growth inhibition hence it inhibited cordycepin production. It is accepted from the reports in literatures that the temperatures for the growth of *Cordyceps* mycelium and exo-polysaccharide production ranged from 20 to 28 °C (Xiao et al., 2004; Park et al., 2001 and Kim et al., 2003) and the production of cordycepin corresponds with secondary metabolite production in other microorganisms, in that the optimum temperature is generally higher than that for growth (Bhosale and Gadre, 2002; Kuznetsov et al., 1984). The result has proved that 25°C is the suitable temperature for cordycepin production but it is not the optimal temperature for cell growth. This could be implied that cordycepin is non-growth associated metabolite.

As mentioned previously, cordycepin of *C. militaris* has an enormous potential for medical and commercial use because of its biological functions, therefore, many efforts have been done in order to meet this demand (Mao and Zhong, 2004; Mao et al., 2005; Masuda et al., 2007). Even though the culture medium and fermentation condition were optimized, as the result, cordycepin yields were very high in these work, however, the yield seems to be not enough for commerce. Many factors

Table 3 Cordycepin production of 15 *C. militaris* strains at four cultivation temperatures after 15 days cultivation[1/].

Fungal strain	Cordycepin production (mg L^{-1})			
	15 °C	20 °C	25 °C	30 °C
C. militaris BCC1974	19.03 ± 0.83a	101.29 ± 21.89b	126.56 ± 20.76b	9.28 ± 0.18a
C. militaris BCC1975	27.79 ± 4.60ab	16.11 ± 1.39ab	36.31 ± 12.89b	1.45 ± 0.16a
C. militaris BCC2790	21.00 ± 0.00a	79.39 ± 14.43b	132.11 ± 17.41c	8.42 ± 0.08a
C. militaris BCC2814	0.00 ± 0.00a	43.80 ± 30.49a	208.27 ± 46.02b	0.25 ± 0.25a
C. militaris BCC2815	1.40 ± 0.81a	7.16 ± 0.10a	187.11 ± 107.61a	0.34 ± 0.03a
C. militaris BCC2816	13.66 ± 8.27a	230.40 ± 29.67b	544.82 ± 99.80c	4.59 ± 0.19a
C. militaris BCC2817	0.00 ± 0.00a	86.54 ± 11.04b	165.92 ± 5.60c	0.40 ± 0.02a
C. militaris BCC2818	0.18 ± 0.00a	7.79 ± 0.60a	161.76 ± 8.39b	1.26 ± 0.09a
C. militaris BCC2819	1.55 ± 0.11a	240.13 ± 20.22b	587.68 ± 16.82c	8.65 ± 0.40a
C. militaris BCC2824	1.60 ± 0.73a	17.23 ± 4.31b	46.99 ± 1.38c	0.66 ± 0.00a
C. militaris BCC2826	0.00 ± 0.00a	184.37 ± 60.86b	109.26 ± 36.86ab	8.24 ± 0.28a
C. militaris BCC2838	0.00 ± 0.00a	22.71 ± 1.22a	42.51 ± 21.21a	0.63 ± 0.19a
C. militaris NBRC5298	0.00 ± 0.00a	0.55 ± 0.02a	0.55 ± 0.03a	0.42 ± 0.39a
C. militaris NBRC9787	1.15 ± 0.06a	8.52 ± 3.66a	104.46 ± 45.45b	0.69 ± 0.43a
C. militaris NBRC100741	1.27 ± 0.15a	26.69 ± 7.96ab	93.81 ± 37.67b	1.17 ± 0.06a

[1/] All values are means of duplicate experiments ± SD. Values with same letters within a row are not significantly different at $P<0.05$.

affected on the commercial production of cordycepin and other metabolite (adenosine) of the fungus *C. militaris* such as the capacity of strains, medium composition, fermentation condition, etc. in which, the selection of high capacity strains for high yield cordycepin production is a very important process. For this reason, the study of Das et al. (2008), followed up the result of Masuda et al. (2007), was successfully in obtaining a higher cordycepin production using *C. militaris* NBRC9787 mutant obtained by "ion beam" mutagenesis technique, this is one of the ways to obtain a target strain. Alternatively, selection of strains with highest cordycepin capacity from available biological resources is very important before going further in advanced technique in order to improve strain to reach a commercial level. The similar strategy for strain improvement could be emoployed with the selected strains from the current research to achieve the cost-effective strains in large scale fermentation.

Conclusions

The growth of *C. militaris* was not consistent with the cordycepin production among different fermentation temperatures. The temperature from 15 ^{o}C to 20 ^{o}C was suitable for mycelium growth, but the optimum temperature for cordycepin production was 25 ^{o}C. Among 15 investigated strains, *C. militaris* BCC2816 and *C. militaris* BCC2819 are excellent cordycepin production strains and they could be used for production purpose.

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