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Contributed Paper

Phenotypic Marking of *Cordyceps militaris* Fruiting-Bodies and Their Cordycepin Production

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ABSTRACT

Cordyceps militaris has recently been used as a functional food in China and is commonly used as a Traditional Chinese Medicine. The formation of *C. militaris* fruiting bodies is unstable and is a limiting factor for industrial production. Fruiting body formation and quantities of adenine, adenosine and cordycepin differed significantly between the phenotypes. Orange chrome colonies produced fruiting bodies more readily, as compared to those of lighter colour. Cordycepin production generally decreased in orange chrome to apricot orange and white colonies. There was, however, no relationship between cordycepin production and colony colour in the slow growing strains. The yields of fruiting body from primary strains were higher than the isolates from colony sector mutations. On the contrary, cordycepin production in most isolates from the colony sector mutations were higher than the primary strains. Therefore isolates from colony sector mutation in *C. militaris* could be used for screening high-yield strains in cordycepin production. Colony colour is one of the markers for detecting fruiting body and cordycepin production in *C. militaris*. The strategies used for screening high-yield strains could have a wide application in fungal biotechnology.

Keywords: *Cordyceps militaris*, cordycepin, fruiting body, phenotypes marking, solid-state fermentation

1. INTRODUCTION

Cordyceps militaris (Fr.) Link has been recently approved as a functional food and Traditional Chinese Medicine (Licence No: Z20030034/35) because it's significant pharmacological activity. *Cordyceps militaris* has long been recognized as a desirable alternative for natural *Ophiocordyceps sinensis* [1], the gathering of which is causing substantial reductions in populations [2]. *Cordyceps militaris* has also been more commonly used as a edible mushroom in China and therefore the market demand for artificial *C. militaris* has increased. Approximately 3,000 tons of dry fruiting

bodies of *C. militaris* are produce annually by solid-state fermentation (Yang Qirui, Shenyang Qianxiang Cordyceps Co., Ltd. pers. comm.).

Cordyceps militaris is rich source of bioactive compounds, such as polysaccharides, cordycepin, adenosine, amino acid, ergosterol, cordycepic acid, superoxide dismutase (SOD), organic selenium, and multivitamins [3,4]. A series of studies have demonstrated that *C. militaris* has many pharmacological functions including anti-inflammatory [5,6], anti-angiogenetic [7], improvement of insulin resistance and insulin secretion [8], anti-fibrotic [9], inhibition of human glomerular mesangial cell proliferation [10], and growth inhibition of U937 leukemia cells [11]. Cordycepin, a nucleoside derivative isolated from the culture medium of *C. militaris* has attracted considerable attention [12] because of its effects as anti-tumor [13], anti-virus [14], and anti-leukemia [15] agents, in the prevention of hypolipidemia [16], in treating and prevention of obesity [17], and as an anti-aging product [18]. Cordycepin is also a Phase I/II clinical stage drug candidate for treatment of refractory Acute Lymphoblastic Leukemia (ALL) patients who express the enzyme terminal deoxynucleotidyl transferase (TdT) (ClinicalTrials. Gov, verified by OncoVista, Inc., 2009). A recent study has shown that many of the reported pharmacological functions of cordycepin are likely to be due to its effects on mTOR (mammalian target of rapamycin) and AMPK (AMP-activated kinase) signaling [19].

Both chemical and biological pathways for the synthesis of cordycepin have been studied, cordycepin is hard to purify via chemical pathways because of the difficulty in maintaining a high standard of the substance and the fact it costs much more than biological pathways [20]. Thus biological pathways have become the major research concern for cordycepin. *In vitro* mycelium growth and cordycepin production by *C. militaris* have attracted considerable research interest by mycologists, entomologists and biotechnologists. There have been various studies on the culture conditions [21,22] and medium composition [4,23,24] for increasing the yield of cordycepin production via liquid culture. However there were only a few reports on solid-state fermentation of *C. militaris*. Fruiting body formation [25-27] and cordycepin production in the medium [28, 29] of *C. militaris* by solid-state fermentation have been reported. Compared to liquid culture, biomass and cordycepin production by solid-state fermentation of *C. militaris* is cheaper and easier.

Solid-state fermentation of *C. militaris* is an efficient way to solve the insufficient availability of the resource in the nature. However in the process of solid-state fermentation culturing of *C. militaris*, the isolates showed unstable variation in forming fruiting body. Most of the isolates failed to produce fruiting-bodies or produced only a few deformed ones. Some isolates initially produced good fruiting-bodies, but the quantity and quality was not maintained in subcultures. Degeneration of isolates in forming fruiting-bodies has become a key restrictive factor in industrial production [27]. Because *C. militaris* can produce much more cordycepin than *O. sinensis* [30], culturing of *C. militaris* with enhanced cordycepin content in its fruiting-bodies would be the best way to meet the increasing demand for cordycepin.

The correlation between morphology and fruiting body formation and cordycepin production in *C. militaris* has rarely been studied. In present study we report on the effect of phenotype of C.

militaris from single ascospores on fruiting body formation and cordycepin production via solid-state fermentation. The results demonstrated that phenotypes could be regarded as one marker of fruiting body formation and cordycepin production. Isolates from colony sector mutations could also be used to screen high-yield cordycepin production strains of *C. militaris*. The results obtained in this work would have an impact on production of fruiting-bodies and cordycepin production via solid-state fermentation at industrial scale.

2. MATERIALS AND METHODS

2.1 Microorganism

Three different wild specimens of *Cordyceps militaris* named 081, 082 and 092 were used in the present study and were collected from Mt. Wawu in Sichuan Province, Yuse National Forest Park in Guizhou Province and Mt. Leigong in Guizhou Province in China. Ascospores were discharged from the fresh specimen on to 2% (w/w) water agar. Single ascospores were picked up one by one using a dissecting needle under a stereomicroscope. Single ascospore plates were numbered in order and any sector mutations transferred to new plates. The letter S in isolates of this investigation correspond to colony sector mutations from the original single ascospore cultures.

Single ascospores strains from the wild specimens were divided into eight different phenotypes (A - H) based on colony color, morphology, growth rate, and with or lacking colony mutation sectors (Figure 1).

2.2 Solid-state Fermentation

The medium for solid-state fermentation was prepared by mixing 20 g of rice and 32 ml of liquid medium (20 g/l sucrose, 10 g/l peptone, 0.1 g/l $MgSO_4 \cdot 7H_2O$ and 0.1 g/l KH_2PO_4 with 1,000 ml distilled water) in 300 ml cylindrical glass bottle and autoclaved for 20 min at 121°C. The medium was cooled to room temperature and inoculated with 5 ml seed culture and incubated at 20°C for 12 d and was given dark treatment for promoting vegetative growth. Primordial fruiting body formation began after 12-15 d after lowering the incubation temperature to 16°C at night in darkness with the culture temperature maintained at 23°C in the day (the light maintained at 500 lx) and relative humidity (RH) at 90%-95%. Then the temperature was maintained at 23°C and RH at 80%-90%; sufficient air changes were maintained to keep the CO_2 concentration at a normal level. Illumination with 300 lx intensity did not exceed 12 hours per day. The culture develops 5-9 cm long fruiting-bodies within 50-60 d after inoculation. All experiments were performed at least in duplicate.

2.3 Analytical Methods

The fruiting bodies and culture medium were dried overnight to a constant weight at 55°C. Cordycepin, adenosine and adenine was analyzed by HPLC (1,100 series, Agilent Technology, U.S.). Standard cordycepin, adenosine and adenine samples from Sigma were dissolved in distilled water for calibration. The mobile phase was 10 mM KH_2PO_4, which was dissolved in methanol/distilled water (6:94). Elution was performed at a flow rate of 1 ml/min with column temperature at 45°C and at a UV wavelength of 259 nm.

3. RESULTS AND DISCUSSION

3.1 Colony Characteristics of Single Ascospore Strains of *C. militaris*

The ascospore germination rates were high, the lowest being from Yuse National Forest Park in Liupanshui (specimen 081,

96.68%) (Table 1). Three main colony types, orange chrome, apricot orange and white, were isolated from single ascospores from the three specimens (Table 1). The apricot orange colonies were most frequent with 80.77%, 68.42%, and 61.68% being from specimens 081, 082 and 092 respectively. Most colonies grew normally with little difference in growth rate. The proportion (7.89%) of slow-growing colonies obtained from specimen 082 was slightly higher than from the other two specimens. There were significant differences in sector mutation occurrence amongst the single ascospores colonies of the three specimens. Specimen 081 had the largest proportion (63.08%) of sector mutation isolates.

Table 1. Comparison of culture characteristics of the populations via single ascospore isolation from the three different wild specimens of *C. militaris* (%).

Specimens	Collection Site	Collection Date	Colony colour			Growth rate		Sector mutation	
			Orange Chrome	Apricot Orange	White	Normal Growth	Slow Growth	Have	None
081	Yuse National Forest Park in Guizhou Province	2008	5.38	80.77	13.85	96.92	3.08	63.08	36.92
082	Mt. Wawu in Sichuan Province	2008	15.79	68.42	15.79	92.11	7.89	34.21	68.79
092	Mt. Leigong in Guizhou Province	2009	11.22	61.68	27.10	95.33	4.67	23.36	76.64

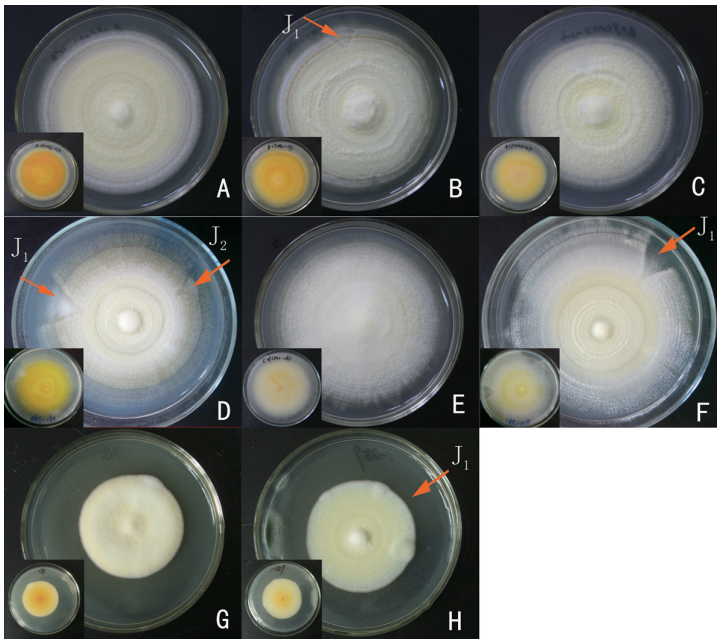

Figure 1. Phenotypes of *C. militaris* single ascospore strains (A: Orange chrome, normal growth, no mutation. B: Orange chrome, normal growth, mutation. C: Apricot orange, normal growth, no mutation. D: Apricot orange, normal growth, mutation. E: White, normal growth, no mutation. F: White, normal growth, mutation. G: White, slow growth, no mutation. H: White, slow growth, mutation).

3.2 The Effect of Phenotypic Polymorphism on Fruiting Body and Cordycepin Production via Solid-state Fermentation of *C. militaris*

Phenotypic polymorphism greatly effected fruiting body formation as well as adenine, adenosine and cordycepin production (Tables 2, 3 and 4). The strains with orange chrome colonies easily produced fruiting-bodies (phenotypes A and B). In the strains with apricot orange colonies fruiting body formation was less common (phenotypes C and D). White colony strains or those growing slowly rarely produced fruiting-bodies (phenotypes E, F, G and H).

Some primary strains (i.e. original single ascospore cultures) produced fruiting-bodies, while sector mutations isolates (the sector mutation part of the original single ascospore cultures, see Figure 1) did not produce fruiting bodies (i.e. F081-180, B081-72 and F082-155). Some primary strains did not produce fruiting-bodies while the isolates from sector mutations produced fruiting-bodies (i.e. B081-85).

The yields of fruiting body from primary strains were higher than the isolates from colony sector mutations. Fully fermented isolates generally produced heavier fruiting-bodies and less dry medium weight.

The fruiting body yield in primary strains was higher than the isolates of sector mutations except for a few isolates. Such as fruit body yield in D081-42, H081-227, F082-103, D082-335 and F092-173 was 39.0%, 234.3%, 26.4%, 27.6%, and 67.1% higher than the isolates of sector mutations, respectively.

About 43.8% of the isolates from sector mutations produced more cordycepin than the primary strains. Cordycepin production in the spent medium of D081-42S1, D081-42S2, H081-227S1, D082-335S1, F082-155S2, and D092-163S1 was 39.0%, 54.6%, 404.9%, 34.4%, 172.8%, 32.4% higher respectively, than the primary strain. Cordycepin production in fruiting bodies of D081-176S1, H081-227S1, F082-155S2, D082-335S1 and D092-163S1 were 233.3%, 88.0%, 135.6%, 225.3% and 69.9% higher respectively, than the primary strain.

Strain instability in filamentous fungi results in permanent changes over time [31]. The production of colony mutations have been recorded in numerous fungal species [31, 32]. Genomic DNA methylation was reported in a sector of *Fusarium oxysporum* Schlecht after successive subculturing [33]. In another example, fungal morphological instability was related to dsRNA virus infection [34].

In this study, significant differences in colony color, colony texture, and sector mutation occurred among different groups of single ascosporic colonies, and this result is in agreement with other reports [35]. Recent research showed that colony sectorization in *Metarhizium anisopliae* (Metsch.) Sorokin is a sign of ageing [36].

Table 2. The effect of phenotypic polymorphism on the production of fruiting body, adenine, adenosine and cordycepin of 081 isolates†.

Isolates	Medium Dry Weight (g)	Fruiting Body Dry Weight (g)	Adenine (mg/g)		Adenosine (mg/g)		Cordycepin (mg/g)	
			Medium	Fruiting body	Medium	Fruiting body	Medium	Fruiting body
A081-200	8.59	1.31	0.49	1.72	0.65	2.40	11.44	20.79
B081-72	9.27	1.58	0.82	0.39	1.06	1.54	13.17	13.68

Table 2. Continued

Isolates	Medium Dry Weight (g)	Fruiting Body Dry Weight (g)	Adenine (mg/g)		Adenosine (mg/g)		Cordycepin (mg/g)	
			Medium	Fruiting body	Medium	Fruiting body	Medium	Fruiting body
B081-72S1‡	9.21	—*	0.86	—	1.01	—	6.20	—
B081-85	10.87	—	1.67	—	1.56	—	9.77	—
B081-85S1	9.30	1.64	0.64	0.87	1.29	1.87	3.27	10.01
C081-16	8.80	1.55	1.11	0.99	1.98	1.04	10.30	5.70
C081-40	7.93	2.02	1.02	1.25	2.09	3.64	9.09	14.97
D081-42	10.19	1.14	2.44	3.78	1.23	0.85	10.65	10.08
D081-42S1	9.16	0.82	1.97	2.48	1.77	1.53	14.80	9.95
D081-42S2	8.58	0.94	1.20	1.35	1.02	0.69	16.47	5.64
D081-176	9.62	0.87	0.73	0.59	1.43	1.42	13.72	10.46
D081-176S1	9.70	0.76	0.62	1.73	0.80	3.89	4.40	34.86
E081-25	8.34	1.08	1.63	0.74	2.30	1.94	8.81	3.42
E081-128	8.12	0.86	1.23	1.64	1.97	1.22	10.38	6.85
F081-180	7.41	1.52	1.64	1.54	2.79	1.13	4.30	14.35
F081-180S1	11.73	—	1.71	—	1.46	—	1.32	—
G081-62	10.35	—	0.88	—	0.75	—	9.23	—
H081-227	11.01	1.17	0.78	0.81	0.99	1.88	5.06	8.39
H081-227S1	7.91	0.35	0.84	1.38	1.10	1.41	25.55	15.77

† Values are mean of triple determinations.

‡ Isolate of B081-72S1 come from the first colony sectorization of B081-72. "S" in isolates number of this investigation was indicated the same mean.

* " — " means no fruiting body.

Table 3. The effect of phenotypic polymorphism on the production of fruiting body, adenine, adenosine and cordycepin of 082 isolates†.

Isolates	Medium Dry Weight (g)	Fruiting Body Dry Weight (g)	Adenine (mg/g)		Adenosine (mg/g)		Cordycepin (mg/g)	
			Medium	Fruiting body	Medium	Fruiting body	Medium	Fruiting body
A082-197	9.47	1.96	0.44	0.89	1.28	0.76	9.25	8.34
A082-317	9.24	1.27	0.78	1.10	1.02	1.34	8.13	7.96
B082-200	9.57	—*	0.74	—	1.87	—	13.24	—
B082-200S1	9.78	—	0.44	—	1.92	—	8.82	—
C082-122	9.85	1.38	1.56	2.24	0.62	2.19	7.88	7.63
C082-208	8.74	1.51	0.41	0.49	1.39	1.20	8.08	7.85
D082-220	13.92	—	0.97	—	1.82	—	7.28	—
D082-220S1	9.44	—	0.39	—	1.33	—	3.50	—
D082-335	9.63	1.62	0.28	0.43	0.74	0.69	7.24	5.93

Table 3. Continued

Isolates	Medium Dry Weight (g)	Fruiting Body Dry Weight (g)	Adenine (mg/g)		Adenosine (mg/g)		Cordycepin (mg/g)	
			Medium	Fruiting body	Medium	Fruiting body	Medium	Fruiting body
D082-335S1	9.27	1.27	1.64	2.09	3.21	3.99	9.73	19.29
E082-94	12.22	—	0.93	—	1.97	—	6.14	—
F082-103	8.64	1.10	1.02	0.65	2.50	2.99	6.23	7.10
F082-103S1	8.55	0.87	0.32	0.49	0.87	0.45	3.11	5.58
F082-155	11.31	1.85	1.21	1.45	1.88	2.23	4.23	7.78
F082-155S1	11.78	—	0.49	—	0.65	—	4.45	—
F082-155S2	12.54	1.22	1.25	2.04	2.55	2.97	11.54	18.33
G082-328	10.21	—	0.52	—	0.39	—	7.62	—
H082-2	13.65	—	3.87	—	4.98	—	6.24	—
H082-2S1	10.86	—	2.16	—	7.29	—	6.92	—

† Values are mean of triple determinations.
* " — " means no fruiting body.

Table 4. The effect of phenotypic polymorphism on the production of fruiting body, adenine, adenosine and cordycepin of 092 isolates†.

Isolates	Medium Dry Weight (g)	Fruiting Body Dry Weight (g)	Adenine (mg/g)		Adenosine (mg/g)		Cordycepin (mg/g)	
			Medium	Fruiting body	Medium	Fruiting body	Medium	Fruiting body
A092-150	9.03	0.31	1.64	1.86	1.93	2.49	8.80	14.77
B092-120	8.67	1.21	0.74	2.81	1.32	3.79	8.03	19.45
B092-120S1	8.27	1.86	0.69	1.08	1.24	2.05	7.95	17.85
C092-43	11.47	0.32	0.97	1.51	0.83	1.87	6.05	15.67
D092-163	8.98	0.70	1.74	3.94	2.96	3.64	8.08	16.30
D092-163S1	8.95	0.52	1.93	1.39	3.10	3.88	10.70	27.69
E092-181	9.66	0.97	0.58	1.51	0.75	1.93	5.45	7.69
F092-173	9.74	1.27	0.81	0.93	0.80	1.35	4.71	6.41
F092-173S1	12.66	0.76	1.33	1.14	1.67	1.19	3.06	5.21
G092-78	7.09	0.48	1.95	2.17	2.30	2.59	9.51	16.26
H092-112	10.28	—*	0.65	—	1.45	—	4.59	—
H092-112S1	11.21	—	0.72	—	0.93	—	5.16	—

† Values are mean of triple determinations.
* " — " means no fruiting body.

3.3 The Effect of Phenotypic Polymorphism on Total Cordycepin Production in Solid-state Fermentation of *C. militaris*

The phenotypic polymorphism greatly affected total cordycepin production (Figures 2-4). Colony colour greatly affected total cordycepin production in *C. militaris* solid-state fermentation. Cordycepin production from single ascospore colonies generally decreased with colony colour (means except isolates

from colony sectorization). Total cordycepin production was greatest in orange chrome colonies, than in apricot orange colonies and least in white colonies. Cordycepin production in slow growing strains (G and H) had no relationship with colony color.

The highest total cordycepin producing isolates (H081-227S1, 207.62±3.92 mg/bottle) were from specimen 081 and were 13.4 times higher than the lowest one (F081-180S1), the cordycepin production in other isolates was mainly about 60-150 (mg/bottle) (Figures 2-4).

It is noteworthy that half of isolates from sector mutations produced higher cordycepin amounts than the primary strains. For example, total cordycepin production in sector mutation strains H081-227S1, F082-155S2, D082-335S1 and D092-163S1 were 3.17, 2.68, 1.45 and 1.64 times higher respectively than the primary strains.

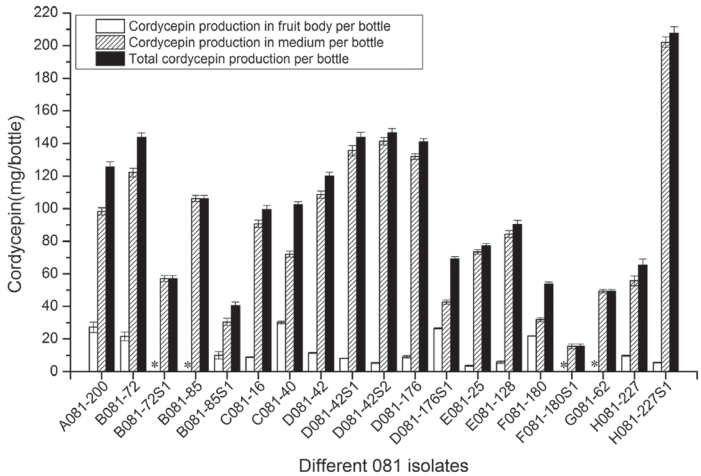

Figure 2. The total cordycepin production of different 081 isolates in SSF (total cordycepin was estimated by cordycepin in culture medium plus cordycepin in fruiting body of one bottle. The results were expressed as the average of triple determinations with S.D.).

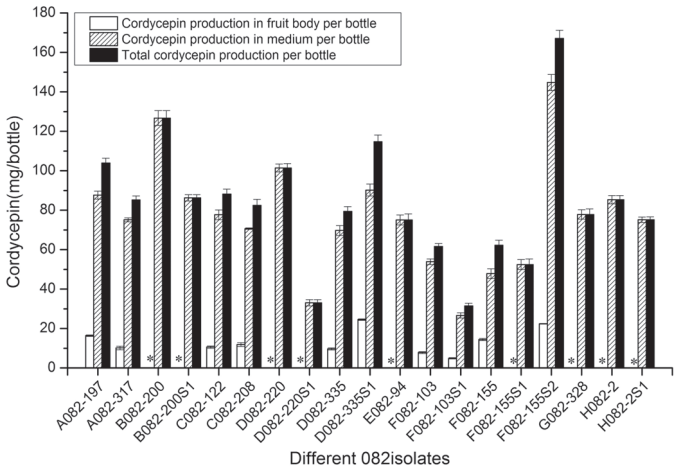

Figure 3. The total cordycepin production of different 082 isolates in SSF (total cordycepin was estimated by cordycepin in culture medium plus cordycepin in fruiting body of one bottle. The results were expressed as the average of triple determinations with S.D.).

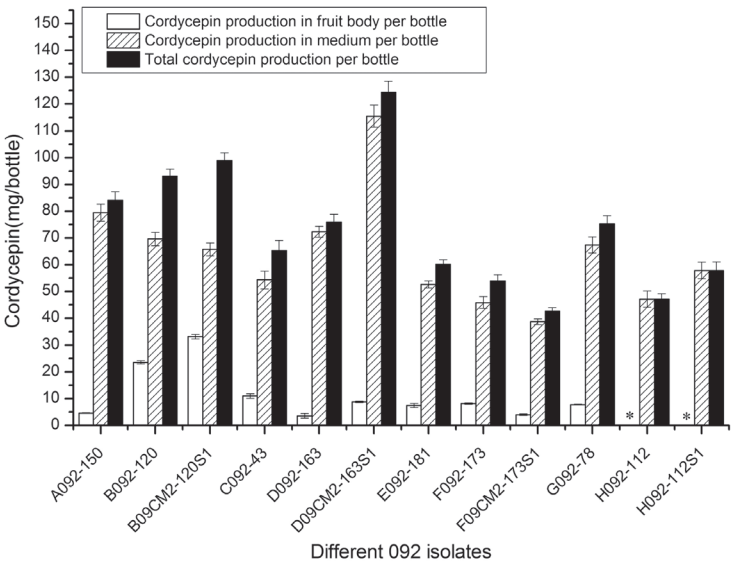

Figure 4. The total cordycepin production of different 092 isolates in SSF (total cordycepin was estimated by cordycepin in culture medium plus cordycepin in fruiting body of one bottle. The results were expressed as the average of triple determinations with S.D.).

Based on the above analysis, phenotypes could be regarded as one of markers (indicator) for fruiting body formation and cordycepin production in *C. militaris*. Also, isolates from sector mutations could be a new way to screen for high-yielding cordycepin producing strains. The results obtained in this work may have a significant impact on industrial scale production of fruiting bodies and cordycepin production using solid-state fermentation. The strategies used for screening high-yield strains could be used in other fungal biotechnological applications.

Sector formation was frequently accompanied by changes in the ability to produce secondary metabolites and enzymes in *M. anisopliae* [32]. The parental strain of *Trichoderma viride* Pers. and three colour mutants (milk white, yellow and brown) blocked at various stages of colony pigmentation derived from it were characterized, they exhibited different growth rates and excreted different anthraquinone pigments [37]. In another case, *Isaria cicadae* Miq. strain JZD3 (the original cultures) produced the highest amounts of exopolysacharides, SOD, POD and the microbial virulence to *Brevicoryne brassicae* (L.). However, the isolates JZD3-C (the spore type sector mutation) and JZD3-M (the hypha type sector mutation) had the highest activity of proteinase and content of proteins [38].

Fungal culture degeneration is usually irreversible and inheritable, and can result in great commercial losses [31, 39]. As a heterothallic fungus, subculturing too frequently can result in the isolation of the heterocaryon (the common reason of colony sectorization) in fungi [40], and this will have a negative influence on the productivity of fruiting body in *C. militaris* [27]. The number of subcultures must be reduced to avoid commercial losses. However since half of the isolates in sector mutations are much higher cordycepin produces than the primary strains; this indicates that sector mutation isolates may be used for screening high-yield strains that can produce secondary metabolites.

4. CONCLUSIONS

The effect of phenotypes on fruiting body and its secondary metabolism of *C. militaris* were investigated in this study. Fruiting body formation and quantities of adenine, adenosine and cordycepin differed significantly between the phenotypes. Orange chrome colonies produced fruiting bodies more readily. Cordycepin production generally decreased in orange chrome to apricot orange and white colonies. Isolates from colony sector mutation in *C. militaris* could be used for screening high-yield strains in cordycepin production. Colony colour is one of the markers for detecting fruiting body and cordycepin production in *C. militaris*. The strategies used for screening high-yield strains could have a wide application in other fungal biotechnology.

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