

Original Research Article

Sequential Strain Improvement of *Cordyceps militaris*

ABSTRACT

Aim -As a highly valued fungus, *Cordyceps militaris* has been widely used all over the world. Although the wild resources of *C. militaris* are limited, the fruiting bodies of *C. militaris* have been successfully cultivated on a large-scale with limited Cordycepin production. However, the high-frequency degeneration of *C. militaris* during subculture and preservation seriously limits the development of the *C. militaris* industry.

Methods and Results-In this study, three different techniques for strain improvement viz., protoplast fusion; UV irradiation and chemical mutagenesis were assessed for improved biomass and cordycepin production.

Conclusion-It was observed that, fusant subsequently mutated were stable even after sub culturing and improved biomass and Cordycepin yields. It was found that fusants after mutagenesis will be genetically stable and this methodology can be used for strain improvement of genetically unstable microorganisms.

Keywords: *Cordyceps militaris*, Cordycepin, Protoplast fusion, Sequential mutagenesis, Fusant

1. INTRODUCTION

Cordyceps militaris is an entomopathogenic fungus, which has been extensively studied for its medicinal uses. *Cordyceps militaris* produces many bioactive compounds, including polysaccharides, cordycepin, adenosine, amino acid, organic selenium, ergosterol, sterols, cordycepic acid, superoxide dismutase (SOD), and multivitamins [1-3]. Cordycepin (3'-deoxyadenosine), a nucleoside analog is one of the most important biologically active metabolites [4]. *C. militaris* has been approved as a functional food and Traditional Chinese Medicine [5]. It is a medicinal agent responsible for immunological regulation [1,6], anticancer [7,8] antifungus [9], antiviral [10], antileukemia [11], anti-diabetic [6,12], antioxidant and antihyperlipidemic [13] activities. Despite of several applications' commercialization of *C. militaris* is difficult due to rare and expensive, since the fungus is host specific and requires strict growth environment [14]. Even wild resources of *C. militaris* are limited; the fruiting bodies of *C. militaris* have been successfully cultivated. However, the high-frequency degeneration of *C. militaris* during subculture seriously limits the development of the *C. militaris* industry [15].

Creation of hybrids and polyploids were found to stabilize strain and rejuvenate metabolite production [16]. Wei-Kuang Lai et al.[17] introduced mutagenesis of protoplast fusants by UV irradiation in fungi. *Cordyceps* mutants from mutagenesis proved to be more efficient than the original strain [18, 19]. Protoplast fusion avoid mating barriers and increase cordycepin production in *Cordyceps Sps* [20]. Protoplast fusion is a feasible method for inter-specific and inter-generic hybridization of edible mushrooms but its yield is poor as compared to non-basidiomycetous fungi [21]. Protoplast fusion with subsequent mutagenesis can produce stable hybrids [22, 23]. Mutant stability can be achieved by sequential mutagenesis [24]. Holliday et al. [25] developed stable hybrid strains of *C. sinensis* using rattlesnake venom.

Wild strains of *C. militaris* are highly unstable [26] and this instability is genetical [15]. There are few studies conducted for strain improvement in *C. militaris* but stable and high cordycepin producing strains for industrial usage are not available. This study focuses on the development of stable and improved strain of *C. militaris* to produce more biomass and Cordycepin.

2. MATERIAL AND METHODS

2.1 Microorganism and Seed Culture

Four different *C. militaris* fruiting bodies were obtained from Shimla, India. The fruiting bodies were washed with sterile normal saline, soaked in 1% sodium hypochlorite solution for 1 minute, and washed again with sterile water thoroughly. The washed fruiting bodies were cut into about 5 mm length and cultured on potato dextrose agar (PDA) potato starch 4 g, glucose 20 g, agar 20 g l⁻¹ medium plates. Three *C. militaris* were isolated (CM1-3) and sub-cultured on PDA. *C. militaris* CM3 was growing very slow hence discontinued in further studies.

The fungi were maintained on potato dextrose agar (PDA) slants. Slants were incubated at 22°C for 7 days and then stored at 4°C. The seed culture was grown in a 250 mL flask containing 100 mL of basal medium (sucrose 20 g l⁻¹; peptone 20 g l⁻¹; KH₂PO₄ 1 g l⁻¹; and MgSO₄·7H₂O 0.5 g l⁻¹) at 22°C on a rotary shaker incubator at 150 rev/min for 5 days.

2.2 Protoplast fusion

Two individual *C. militaris* strains (CM1 and CM2) were inoculated in 50 ml medium (Glucose 80g l⁻¹; NH₄NO₃ 2g l⁻¹; KH₂PO₄ 10g l⁻¹; MgSO₄·7H₂O 0.25g l⁻¹; FeCl₃·6H₂O 0.02g l⁻¹; MnSO₄ 0.014g l⁻¹; pH 4.5) separately and incubated for 36 hr at 20°C on rotatory shaker (150 rpm). The mycelium formed was recovered by centrifugation 12,000 rpm for 10 min, the mycelium was washed twice with sterile physiological saline (0.85% NaCl) and resuspended in 5 ml protoplasting buffer (0.1 M; pH 6) containing 0.7 M NaCl; 0.2 M CaCl₂ and Novozymes 234 (20mg ml⁻¹) and incubated at 20°C with gentle shaking up to 6 hr. Then equal volume of suspension from two parents were mixed and centrifuged. The protoplast pellet formed was resuspended in 2 ml fusion buffer (Phosphate buffer 0.05 M; pH 7.5, 30% w/v PEG 6000, 50 mM CaCl₂ and 0.7 M NaCl.) and incubated for 20 min at 20°C and the suspension was inoculated on PDA media plates.

2.3 UV irradiation

Fusants selected were mutated by UV irradiation. Sporulation was induced by growing Fusant strains on PDA plates with 0.1% Calcium chloride at 20°C. The conidial suspension was prepared in 0.85% NaCl containing 0.1% Tween 80. The suspension was treated with 500µg ml⁻¹ sodium nitrate and was irradiated of 30 min under UV lamp at the distance of 30 cm. After irradiation the conidial suspension was incubated at 20°C for 60 min in dark. Then 0.1 ml suspension was poured on Potato Dextrose Agar and incubated at 20°C for six days until the fungal colonies were observed. The isolates showing rapid growth and the altered morphology were selected.

2.4 Strain improvement by chemical mutagenesis

Out of five mutants obtained after UV mutagenesis, CMF-U5 was found to be potent strains for biomass and cordycepin production and this strain was used for further strain improvement by chemical mutagenesis. Two methods of chemical mutagenesis were employed for strain improvement.

Method I.

Ethidium bromide (EtBr) was used for mutations. Conidia from 1-week-old potato dextrose agar (PDA) plates were suspended in sterile normal saline solution. A suspension containing 5 X 10⁸ conidia ml⁻¹ was treated with 100 µg ml⁻¹ of EtBr for 30 min at 22°C in an orbital shaker. After treatment, conidia were plated on Potato Dextrose agar medium.

Method II.

Mutagen (EtBr) was incorporated in the sterile Potato Dextrose agar medium in sub lethal concentration ($5 \mu\text{g ml}^{-1}$ of EtBr as this concentration was giving 10 to 20 colonies). Spore suspension (0.1 ml) was plated on Potato Dextrose agar plates containing suspended mutagen as above and incubated at 22°C for 10 days.

2.5 Screening

The strains were screened for biomass and cordycepin production; all strains were inoculated in Potato dextrose broth pH6.0 and analyzed the production of mycelial biomass and Cordycepin. Cordyceps culture disc measuring 1cm diameter of selected cultures were inoculated into PDB mediums and incubated at 20°C at pH 6.0 for two weeks with initial four days under shaking. After cultivation in liquid PDB, the mycelial biomass was pelleted by centrifugation at 10,000 rpm for 10 min. Dried at 65°C and weight of mycelia was recorded. The cordycepin was extracted from the liquid phase and powdered mycelium or fruiting bodies in double distilled water under sonication for 3 hrs. Cordycepin was analyzed by HPLC according to Wen et al. [2].

2.6 Seed Culture of *C. militaris* parent and Fusant mutant CP5

C. militaris parent and Fusant mutant CP5 spores (about 1.0×10^8 spores ml^{-1}) from PDA slant agar medium were transferred, into the broth seed medium containing Potato Dextrose Broth (PDB; 4 gl^{-1} potato starch and 20 gl^{-1} glucose) separately. The seed culture was performed at 22°C for 4 days in shaking incubator (200 rpm) with a 250 mL Erlenmeyer flask containing 100 mL of broth seed medium

2.7 Liquid Cultivation

The compositions of liquid medium was as follows: pH 6, 10 gl^{-1} yeast extract, 10 gl^{-1} peptone, 20 gl^{-1} Sucrose, 5 gl^{-1} Lactose, 0.1 gl^{-1} KH_2PO_4 , 0.2 gl^{-1} $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, and 0.2 gl^{-1} $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. The 4% seed broth of *C. militaris* CP5 was transferred into the liquid medium, and the cultivation was performed at 22°C for 25 days in a shaking incubator at 200 rpm in a 250 mL Erlenmeyer flask containing 100 mL of medium. The samples were collected at the end of the fermentation from the flasks for analyzing mycelium biomass dry weight and cordycepin production.

Samples collected were centrifuged at 5000 rpm for 20 min. The mycelium at the bottom of tubes was washed with distilled water and dried to a constant dry weight at 65°C .

For analysis of extracellular cordycepin, the resulting culture filtrate was obtained by centrifugation at 5000 RPM for 10 min. The supernatant was filtered through a $0.45 \mu\text{m}$ membrane and the filtrate was analyzed by HPLC

2.8 Solid Cultivation for Fruiting Body

Solid rice media containing 20g brown rice, 5g Finger millet and 40ml nutrition broth (containing 0.2 gl^{-1} sucrose, 0.1 gl^{-1} peptone, 0.01 gl^{-1} KH_2PO_4 , 0.01 gl^{-1} MgSO_4 , 0.001 gl^{-1} Vitamin B1, 1 multivitamin tablet) prepared in 1000 mL glass jars (inner diameter 110 mm, height 150 mm). All bottles were sealed with polypropylene caps with nonabsorbent cotton plugs and sterilized at 121°C for 30 min.

Inoculation and fruit body cultivation

Seed culture was inoculated evenly into each bottle at the rate of 10 mL per bottle. All cultures were incubated for 10 days in the dark at 20°C with 60–70% relative humidity. When mycelia grew over the medium surface, the incubating room was illuminated by fluorescent light at an intensity of 5000 lux for 12 h per day. Meanwhile, ventilation for a 30 min period was provided every day. The cultivation conditions were maintained for 30 days until the tops of fruiting bodies became round and were covered with spores. After maturation, fresh fruit bodies were collected. At least nine replicates were carried out for each treatment.

Fruit bodies obtained were dried to constant weight at 65°C . Dried samples were pulverized to a particle size of less than 20-mesh. For cordycepin extraction, 0.5g of dry powder was suspended in 50 mL of double deionized water and sonicated for 3 h in an ultrasonic bath at

50 kHz and 400 W. The supernatant obtained by centrifugation at 5000 for 5 min was filtered through a 0.45 μm membrane filter.

2.9 Stability studies of mutants

Fusants, Mutants obtained by the UV and two chemical methods were studied for their stability for biomass and cordycepin production for 20 generation. Mutants were sub-cultured in PDB and PDA and tested for biomass and cordycepin production.

2.10 Analytical Methods

Estimation of cordycepin by HPLC

The cordycepin content of the samples was determined by HPLC (Agilent Technologies Inc., Santa Clara, CA, USA). A 100mg Cordyceps dried and grinded powder was suspended in 100 ml mobile phase and ultrasonicated for 30 minutes. This solution was centrifuged, pre filtered with 0.45- μm membrane filter and used for HPLC. HPLC was carried out with Discovery C18 Column (250 \times 4.6 mm; 5 μm); mobile phase, methanol: water (15:85, v/v); flow rate, 0.8ml min^{-1} ; UV detection, 260 nm; and injection quantity, 10 μl . Quantitative analysis of cordycepin was carried out by evaluating the peak area with standard curve.

2.11 Statistical Analysis

Statistical Analysis of the study was done. Dry weight and cordycepin production are expressed as means \pm SD.

3. RESULTS

3.1 Protoplast fusion

The re-suspended spores in protoplasting buffer formed clumps under microscope. After fusion white colored strains of combination were observed and selected for further studies (Fig 1). Five fused strains (CMF-1 to CMF-5) were selected for comparative analysis based on high biomass production. Generally fusants are unstable, hence further mutagenesis with UV and Chemical mutagenesis is attempted.



Fig-1 *Cordyceps militaris* Fusant-CMF5 grown on PDA plate.

3.2 UV-irradiation

After incubation for seven days, five colonies were selected based on larger size from each fusant and was inoculated in 150ml of PDB broth and incubated for 10 days at 20°C. After incubation the culture was grown in PDA plates. Two UV mutants from each fusant parent were selected for further analysis. It was observed that the CMF5U-1 isolate showed largest colony size in 3 days time (Fig. 2).



Fig- 2 *Cordyceps militaris* Fusant and UV mutant strains CMF 5U-1 grown on PDA plate.

3.3 Chemical Mutagenesis

Mutant CMF5U1 was further mutated with Ethidium bromide by two methods. From exposure method (Method I) and incorporation method (Method II) six mutants each were selected based on colony size and variations in colony morphology. These mutants were further grown in liquid and solid static fermentation and analyzed for biomass and cordycepin production (Table 1).

TABLE -1 Biomass and Cordycepin production of parent and improved strains of *Cordyceps militaris*

Strain development	Strain/Mutant	Liquid fermentation		Solid fermentation
		Mycelium dry weight mgL ⁻¹	Cordycepin produced gL ⁻¹	Cordycepin produced mgg ⁻¹ dry fruiting body
Parent 1	CM1	200±05	2.1±0.15	0.25±0.08
Parent 2	CM2	150±08	3.2±0.18	0.30±0.05
Protoplast fusion	CMF1	450±15	4.2±0.11	0.28±0.11
	CMF2	525±11	4.5±0.10	0.28±0.08
	CMF3	500±21	4.8±0.15	0.30±0.10
	CMF4	575±22	2.8±0.28	0.25±0.15
	CMF5	650±15	5.2±0.17	0.35±0.10
UV Mutagenesis	CMF5-U1	700±10	4.8±0.12	0.28±0.11
	CMF5-U2	500±17	5.1±0.22	0.32±0.20
	CMF5-U3	610±22	5.0±0.10	0.30±0.19
	CMF5-U4	725±11	5.8±0.14	0.40±0.15
	CMF5-U5	890±08	6.5±0.21	0.52±0.20
Method I chemical mutagenesis. (Exposure of mutagen to ascospores)	CMF5U5-1 CP1	560±05	325±0.18	0.42±0.10
	CP2	625±12	320±0.11	0.47±0.15
	CP3	510±17	400±0.20	0.40±0.11
	CP4	425±20	420±0.22	0.45±0.18
	CP5	940±15	8.4±0.21	0.76±0.12
	CP6	570±09	8.1±0.24	0.71±0.10
Method II chemical mutagenesis. (Incorporation of mutagen in media and plating ascospores)	CP7	640±15	8.0±0.18	0.80±0.15
	CP8	685±05	7.8±0.10	0.75±0.11
	CP9	805±09	8.1±0.14	0.68±0.15
	CP10	740±11	7.5±0.12	0.80±0.10
	CP11	1010±15	11.5±0.16	0.91±0.12
	CP12	890±25	9.7±0.20	0.75±0.18

3.4 Characterization of Fusant mutant CP11.

Fusant mutant CP11 was obtained by second method of chemical mutagenesis (Incorporation of Ethidium bromide at sub-lethal concentration in the media) of mutant CMF5U1. There was a higher biomass (1010mg l^{-1}), cordycepin production in liquid (11.5g l^{-1}) and solid fermentation (0.91g g^{-1}) in the fusant mutant CP11 (Fig. 3) as compared to parental culture, fusants, fusant-irritant mutants and other fusant-irritant and chemical mutants developed. Fusant mutant CP11 showing colony morphology similar to parent in PDA plates but mycelium and fruiting bodies are formed in 25 days time compared to 35 days in parent strain. Fusant mutant CP11 fruiting bodies are larger in size with dark orange color. In comparison the developed strain showed higher biomass and cordycepin production.



Figures 3 *Cordyceps militaris* Fusant and mutant strain CP11 grown on PDA plate.

4. DISCUSSION

Wild *Cordyceps militaris* strains were isolated with limited cordycepin productivity. Wild strains were protoplast fused and sequential mutated and improved biomass and cordycepin production. Shrestha et al.[27] mated two strains of *C. militaris* and showed the potential improvement in cordycepin production. Protoplast fusion is preferable when mating strains are not available, hence attempted. Hybrid fusants obtained were stabilized by Holliday et al. [25] using Snake venom. Mani et al [28] used Arbin a plant poison and the developed stable strains with improved cordycepin production. A similar effective protoplast fusion and its stabilization with sequential mutagenesis was observed in the present study.

The present study supports the research done by Zhou& Bian [29] which showed increased cordycepin production and genetic stability in plasma fused strains of *C. militaris*. Moreover Guo et al. [30] successfully fused *C. sinensis* and *C. militaris* and showed morphological and genetically similar to the parent strains. In both studies of plasma fusion heat and ultraviolet inactivation was done to stabilize the fusion but in the present study inactivation procedure was followed to stabilize the fusion with UV and chemical mutagenesis. It was observed that the isolates were stable even after sub-culturing for 20 generations and the colonies were having similar characteristics. The use of low-energy ion beam [31] and proton beam irradiation increased the production of cordycepin[32] The present study showed that UV irradiation can be used for strain development of Cordyceps. In our earlier studies [23] treatment of mutagen continuously at sub lethal concentrations has given improved strain and these mutants were found to be stable compared to mutants obtained by treatment of mutagen for few hours at higher concentrations and plating.

Cordycepin production from $0.25, 0.30\text{mg g}^{-1}$ in wild parent strains increased to 0.910 mg g^{-1} in fruiting bodies whereas in liquid fermentation it was increased from $2.1, 3.2\text{g l}^{-1}$ to 11.5g l^{-1} . Cordycepin produced in solid fermentation by fruiting bodies was 0.81 mg g^{-1} [33]. Masuda M et al [34] reported maximum of 14.3g l^{-1} cordycepin production when *C. militaris* G81-3,

the mutant obtained by a proton beam irradiation, was cultivated by liquid surface culture, where as Li w et al (2009) reported 11.9g/l-1 cordycepin yield in liquid culture. Three techniques employed for strain improvement and improved strain produced was stable and showed improved productivity. This study showed that the sequential strain improvement is effective in developing improved strain of *C. militaris* and can be further utilized to develop effective strains for metabolite and biomass production. Moreover this approach can be evaluated in other fungal species of unstable genetic nature

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