Journal of Advances in Microbiology

Volume 23, Issue 3, Page 13-20, 2023; Article no.JAMB.96982
ISSN: 2456-7116

Sequential Strain Improvement of *Cordyceps militaris*

L. B. Laxmikanth Rathod [a,b], Shaik Muzammil Pasha [a], Anju Rajan [a], Madhavi [a,c] and Chand Pasha [a*]

[a] *Department of Microbiology, Nizam College, Osmania University, Hyderabad, India.*
[b] *Department of Physical Education, Palamuru University, Mahbubnagar, India.*
[c] *Department of Zoology, Osmania University, Hyderabad, India.*

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JAMB/2023/v23i3711

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/96982

Original Research Article

Received: 18/12/2022
Accepted: 24/02/2023
Published: 27/02/2023

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 the fusant subsequently mutated were stable for many generation and yielding improved biomass and Cordycepin. 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.

**Corresponding author: E-mail: cpasha21@gmail.com;*

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], antivirus [10], antileukemia [11], anti-diabetic [6,12], antioxidant and antihyperlipidemic [13] activities. Despite several applications' commercialization of C.*militaris* is difficult due to its rare and expensive, since the fungus is host specific and requires a 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 more efficient than the original strain [18,19] using protoplast fusion to 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 rattle snake 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 developing stable and improved strain of *C. militaris* to produce more biomass and cordycepin.

2. MATERIALS 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 lengths and cultured on potato dextrose agar (PDA) potato starch 4 g, glucose 20 g, agar 20 gl^{-1} medium plates. Three *C. militaris* were isolated (CM1-3) and sub-cultured on PDA. *C. militaris* CM3 was growing very slowly and 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 gl^{-1}; peptone 20 g l^{-1}; KH_2PO_4 1 gl^{-1}; and $MgSO_4{\cdot}7H_2O$ 0.5 gl^{-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}; NH4NO$_3$ 2g l^{-1}; KH_2PO_4 10gg l^{-1}; $MgSO_4{\cdot}7H_2O$ 0.25g l^{-1}; $FeCl_3{\cdot}6H_2O$ 0.02g l^{-1}; $MnSO_4$ 0.014g l^{-1}; pH 4.5) separately and incubated for 36 hr at 20ºC 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 20ºC 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 $CaCl_2$ and 0.7 M NaCl.) and incubated for 20 min at 20ºC and the suspension was inoculated on PDA media plates" [27].

2.3 UV Irradiation

Fusants selected were mutated by UV irritation. Sporulation was induced by growing fusant strains on PDA plates with 0.1% Calcium chloride at 20ºC. "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 20ºC 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". [27] 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 strain 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 10^8 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 µg 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 sporesml^{-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, 5gl^{-1}Lactose, 0.1 gl^{-1} KH_2PO_4, 0.2 $gl^{-1}$$K_2HPO_4{\cdot}3H_2O$, and 0.2 gl^{-1} $MgSO_47H_2O$.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 *µ*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 0.2gl^{-1} sucrose, 0.1gl^{-1} peptone, 0.01gl^{-1} KH_2PO_4, 0.01gl^{-1} $MgSO_4$, 0.001gl^{-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.

2.8.1 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 10days 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

2.10.1 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 (250×4.6 mm; 5 μm); mobile phase, methanol: water (15:85, v/v); flow rate, 0.8ml min^{-1}; UV detection, 260 nm; and injection quantity, 10μl. 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 ± 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).

Table 1. Biomass and cordycepin production of parent and improved strains of *Cordyceps militaris*

		Liquid fermentation		Solid fermentation
Strain development	**Strain/Mutant**	**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

3.3 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).

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 (1010mgl^{-1}), cordycepin production in liquid (11.5gl^{-1}) and solid fermentation (0.91gg^{-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 CP11showing 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.

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. [28] 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 [27] 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 were observed in the present study.

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

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

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 were 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, 0.30mgg^{-1} in wild parent strains increased to 0.910 mgg-1 in fruiting bodies whereas in liquid fermentation it increased from 2.1, 3.2gL^{-1} to 11.5gl$^{-1.}$ Cordycepin produced in solid fermentation by fruiting bodies was 0.81 mg g-1 [33]. Masuda M et al. [34] reported maximum of 14.3gl-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 11.9gl-1 cordycepin yield in liquid culture.

5. CONCLUSION

The mutant CP11 obtained by three techniques of strain improvement was stable and showed improved productivity. This study showed that the sequential strain improvement is effective in developing an 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.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Lee CT, Huang KS, Shaw JF, Chen JR, Kuo WS, Shen G, Grumezescu AM, Holban AM, Wang YT, Wang JS, Hsiang YP, Lin YM, Hsu HH, Yang CH. Trends in the immunomodulatory effects of *Cordyceps militaris:* total extracts, polysaccharides and cordycepin. Front Pharmacol 2020;11:575704.
2. Wen T, Kang J, Hyde KD, Li G, Kang C, Chen X. Phenotypic marking of *Cordyceps militaris* fruiting-bodies and their cordycepin production. Chiang Mai Journal of Science. 2014;41:846–857.
3. Jedrejko KJ, Lazur J, Muszynska B. *Cordyceps militaris*: An overview of its

chemical constituents in relation to biological activity. Foods. 2021;30:10(11): 2634.

4. Phull AR, Ahmed M, Park HJ. *Cordyceps militaris* as a bio functional food source: pharmacological potential, anti-inflammatory actions and related molecular mechanisms. Microorganisms. 2022;10(2):405.
5. Wen T, Li G, Kang J, Kang C, Hyde KD. Optimization of solid-state fermentation for fruiting body growth and cordycepin production by *Cordyceps militaris*. Chiang Mai Journal of Science. 2014;41:858–872.
6. De Silva DD, Rapior S, Hyde KD, Bahkali AH. Medicinal mushrooms in prevention and control of diabetes mellitus. Fungal Diversity. 2012;56(1):1–29.
7. Yoshikawa N, Yamada S, Takeuchi C, et al. Cordycepin (3′-deoxyadenosine) inhibits the growth of B16-BL6 mouse melanoma cells through the stimulation of adenosine A3 receptor followed by glycogen synthase kinase-3*β* activation and cyclin D1 suppression. Naunyn-Schmiedeberg's Archives of Pharmacology. 2008;377:4–6,591–595.
8. Reis FS, Barros L, Calhelha RC, Ciric A, Van Griensven LJ, Sokovic M, Ferreira IC. The Methanolic Extract of *Cordyceps militaris* (L.) link fruiting body shows antioxidant, antibacterial, antifungal and antihuman tumor cell lines properties. Food Chem Toxicol. 2013;62: 91–98.
9. Sugar AM, McCaffrey RP. Antifungal activity of 3'-deoxyadenosine (cordycepin). Antimicrobial Agents and Chemotherapy. 1998;42(6):1424–1427.
10. Hashimoto K, Simizu B. Effect of cordycepin on the replication of western equine encephalitis virus. Archives of Virology. 1976;52(4):341–345.
11. Kodama EN, McCaffrey RP, Yusa K, Mitsuya H. Antileukemic activity and mechanism of action of cordycepin against terminal deoxynucleotidyl transferase-positive (TdT^{+}) leukemic cells. Biochemical Pharmacology. 2000;59(3):273–281.
12. Dong Y, Jing T, Meng Q, Liu C, Hu S, Ma Y, Liu Y, Jiahui Lu, Yingkun Cheng, Di Wang, Lirong Teng. Studies on the antidiabetic activities of cordyceps militaris extract in diet-streptozotocin-induced diabetic sprague-dawley rats. BioMed Research International. 2014;1:160980.
13. Ahn HY, Cho HD, Cho YS. Anti-oxidant and anti-hyperlipidemic effects of cordycepin-rich *Cordyceps militaris* in a Sprague–Dawley rat model of alcohol-induced hyperlipidemia and oxidative stress. Bioresour Bioprocess 2020;7:33.
14. Lee SK, Lee JH, Kim HR, Chun Y, Lee JH, Yoo HY, Park C, Kim SW. Improved cordycepin production by *Cordyceps militaris* KYL05 using casein hydrolysate in submerged conditions. Biomolecules. 2019;9(9):461.
15. Lou H, Lin J, Guo L. Advances in research on *Cordyceps militaris* degeneration. Appl Microbiol Biotechnol. 2019;103(19):7835-7841.
16. Chen A, Wang Y, Shao Y, Huang B. A Novel technique for rejuvenation of degenerated caterpillar medicinal mushroom, *Cordyceps militaris* (Ascomycetes), a Valued Traditional Chinese Medicine. Int J Med Mushrooms. 2017;19(1):87-91.
17. Wei Kuang Lai, Yang Chang Wu, Tai Sheng Yeh, Chun Ren Hsieh, Yi Hong Tsai, Chien Kei Wei, Chi Ying Li, Ying Chen Lu, Fang Rong Chang. The protoplast two-way fusions and fusant characteristics of *Antrodia cinnamomea* and *Cordyceps militaris.* Food Science and Human Wellness. 2022;11(5):1240-1251.
18. Huang SJ, Lin CP, Mau JL, Li YS, Tsai SY. Effect of UV-B Irradiation on Physiologically active substance content and antioxidant properties of the medicinal caterpillar fungus *Cordyceps militaris* (Ascomycetes). Int J Med Mushrooms. 2015;17(3):241-253.
19. Das SK, Masuda M, Hatashita M, Sakurai A, Sakakibara M. Optimization of culture medium for cordycepin production using *Cordyceps militaris* mutant obtained by ion beam irradiation. Process Biochem. 2010;45:129–132.
20. Guo Chengjin, Zhao Run. Study on protoplast preparation and regeneration of *Cordyceps sinensis*. J Food Science. 2009;30(5):166-170.
21. Eyini M, Rajkumar K, Balaji P. Isolation, regeneration and PEG-induced fusion of protoplasts of *Pleurotus pulmonarius* and *Pleurotus florida*. Mycobiology. 2006;34(2): 73-8.
22. Zaidi and Kamal. An effective approach of strain improvement in *Cordyceps militaris* using abrin. Current Research in

Environmental & Applied Mycology. 2016; 6:166-172.

23. Pasha C, Kuhad RC, Rao LV. Strain Improvement of thermotolerant *Saccharomyces cerevisiae* VS3 strain for better utilization of lignocellulosic substrates. Journal of Applied Microbiology. 2007;103:1480–1489.
24. Pasha C, Aruna A, Maqsood AM, Rao LV. Novel mutation method for increased cellulase production. Journal of Applied Microbiology. 2005;98:318–323.
25. Holliday JC, Cleaver P, Loomis Powers M, Patel D. Analysis of quality and techniques for hybridization of medicinal fungus Cordycepsinensis (Berk.) Sacc. (Ascomycetes). International Journal of Medicinal Mushrooms. 2004;6:151–164.
26. Liu Q, Wang F, Liu K, Dong C. Influence of strain preservation methods on fruiting body growth and metabolite production by the medicinal mushroom *Cordyceps militaris* (Ascomycetes). Int J Med Mushrooms. 2018;20(10):1003-1011.
27. Mani A, Thawani V, Zaidi KU. An effective approach of strain improvement in *Cordyceps militaris* using abrin. Current Research in Environmental & Applied Mycology. 2016;6(3):166–172.
28. Shrestha B, Han SK, Yoon KS, Sung JM. Morphological characteristics of conidiogenesis in *Cordyceps militaris*. Mycobiology. 2005;33:69-76.
29. Zhou HY, Bian YB. Identification and selection of high cordycepin-yielding protoplast fusion products of *Cordyceps militaris.* Acta Edulis Fungi. 2007;4:69–74.
30. Guo CJ, Zhao R, Zhu WB. Protoplast fusion between *Cordyceps sinensis* and *Cordyceps militaris*. Food Science. 2010;31:165–171.
31. Li W, Zhao S, Chen H, Yuan H, Wang T, Huang X. High-yielding cordycepin in *Cordyceps militaris* modified by low-energy ion beam. Sheng Wu, Gong Cheng, Xue Bao. 2009;25(11):1725-31.
32. Das SK, Masuda M, Hatashita M, Sakuraiand A, Sakakibara M. A new approach for improving cordycepin productivity in surface liquid culture of *Cordyceps militaris* using high-energy ion beam irradiation. Letters in Applied Microbiology. 2008;47:534–553.
33. Jedrejko K, Kała K, Sułkowska-Ziaja K, Krakowska A, Zieba P, Marzec K, Szewczyk A, Sekara A, PytkoPolonczyk J, Muszynska B. *Cordyceps militaris*—Fruiting bodies, mycelium, and supplements: valuable component of daily diet. Antioxidants. 2022;11:1861.
34. Masuda M, Das SK, Hatashita M, Fujihara S, Sakurai A. Efficient production of cordycepin by the *Cordyceps militaris* mutant G81-3 for practical use. Process Biochem. 2014;49:181–187.

© 2023 Rathod et al.; This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Peer-review history:
The peer review history for this paper can be accessed here:
https://www.sdiarticle5.com/review-history/96982