



## An effective approach of strain improvement in *Cordyceps militaris* using abrin

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Mani A, Thawani V, Zaidi KU 2016 – An effective approach of strain improvement in *Cordyceps militaris* using abrin. Current Research in Environmental & Applied Mycology 6(3), 166–172, Doi 10.5943/cream/6/3/3

### Abstract

*Cordyceps militaris*, a medicinal fungus facing conservation from excessive harvesting and specific environment need for growth. In this study, three different techniques for strain improvement viz., somatic fusion, protoplast fusion and UV irradiation were assessed for biomass, exo-polymer and cordycepin production. It was observed that, somatic hybridization was more effective and the developed hybrids were stable even after sub culturing. Moreover hybrids developed by protoplast fusion were stable rather U-V irradiation mutant showed characteristic changes after sub culturing. In the study abrin was used to trigger somatic fusion and it was found to be an effective approach in strain improvement.

**Key words** – Biomass – Cordycepin – Exo-polymer – plasma fusion – somatic hybridization – UV irradiation

### Introduction

*Cordyceps militaris* (Fr.) Link is an entomo-pathogenic fungus, which has been extensively studied for its medicinal uses. The myriad of its health benefits include immunomodulatory, hypoglycemic, hypolipidemic, and antitumor activity (Russell & Paterson 2008, Zhou et al. 2009, Das et al. 2010, Lin & Li 2011). *C. militaris* has been recently approved as a functional food and Traditional Chinese Medicine by Chinese government (Wen et al. 2014a). In the nature *C. militaris* rare and expensive, since the fungus is host specific and requires strict growth environment, hence its commercial utilization is a tough issue (Patel & Ingahlalli 2013). This study was directed at strain improvement for metabolite and biomass production. Protoplast fusion is a known to be a feasible method for inter-specific and inter-generic hybridization edible mushrooms (Aswini et al. 2014) but its yield is poor as compared to non-basidiomycetous fungi (Gupta et al. 1997). Hybridization and Protoplast fusion can produce hybrids with the combined properties of both parents (Zhao & Chang 1995, Feng et al. 2012). Holliday et al. (2004) developed hybrid strains of *C. sinensis* using rattlesnake venom by triggering somatic fusion which resulted in reproducible strain containing qualities of recognized bioactive compounds. The effect of UV light on vegetative compatibility in the fungus and developed the procedure to detect individual, incompatible colonies from cultured lawns of compatible colonies (Rizwana & Powell 1992). Zhao et al. (2011) introduced inactivated protoplast fusion by UV

irradiation in fungi. Mutants from these techniques proved to be more efficient than the original strain (Alberts et al. 2002). There are no studies conducted for strain improvement in *C. militaris* even though this fungus has good industrial application. This study focuses on the development of hybrids to produce more potent metabolite from the two strains of *C. militaris*.

## Materials and Methods

### Micro-organism and Medium

*C. militaris* (MTCC 3936) and *C. militaris* wild strain (CCM 1) was cultured in Corn Meal Agar (CMA) at 20°C for two weeks. For mycelia culturing Potato malt Peptone (PMP), medium pH 6.0 at 20°C on shaking condition was used for six days. Then the culture flasks were incubated at 20°C for 10-15 days, without shaking.

### Strain Development

#### Hybridization

Abrin was extracted by soaking 10 g pulverized seeds of *Abrus precatorius* in PBS (pH 7.7) with 5 % NaCl for overnight at 4°C. The homogenate was filtered and centrifuged at 10,000 rpm for 30 min at 4°C. The crude abrin was dialyzed against distilled water against 10 mM Tris-HCl pH 7.7 for 24 h each. The dialyzed solution was centrifuged at 15,000 for 20 min, and the partially purified abrin was used for hybridization.

Corn meal agar (CMA) plates were prepared and inoculated with two strains of *C. militaris* viz. MTCC 3936 and CCM-1, on opposite sides of the Petri-plate and were incubated at 20°C. After one week of incubation, a furrow was made in the middle of the plate of 7cm X 0.2cm and 100 µl of crude abrin (20µg/ml total protein) was added. The plates were again incubated at 20°C for more than one week till mycelial growth was seen, when the stromata were initiated at the meeting line between the strains, and were isolated into a new CMA.

#### Protoplast fusion

The spores were collected by centrifuging of filtered liquid culture at 10,000 rpm for 10 min. The spore suspension of each parent was inoculated in 50 ml protoplasting medium (Glucose 80g/L; NH<sub>4</sub>NO<sub>3</sub> 2g/L; KH<sub>2</sub>PO<sub>4</sub> 10g/L; MgSO<sub>4</sub>·7H<sub>2</sub>O 0.25g/L; FeCl<sub>3</sub>·6H<sub>2</sub>O 0.02g/L; MnSO<sub>4</sub> 0.014g/L; pH 4.5) and incubated for 36 hr to 20°C on rotatory shaker (180 rpm). The mycelium formed was recovered by centrifugation 12,000 rpm for 10 min, the mycelium was washed twice with sterilized physiological saline (0.85% NaCl) and resuspended in 5 ml protoplasting buffer (0.1 M; pH 5.8-6) containing 0.7 M NaCl; 0.2 M CaCl<sub>2</sub> and Novozyme 234 (20mg/ml) and incubated at 20°C with gentle shaking for 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<sub>2</sub> and 0.7 M NaCl.) and incubated for 20 min at 20°C and the suspension was inoculated on CMA media.

#### UV irradiation

Sporulation was induced by growing strains on CMA plates at 20°C and subcultured. After subculturing, conidial suspension was prepared 0.85% NaCl containing 0.1% Tween 80. The suspension was treated with 500µg/ml sodium nitrate and was irradiated of 80 min under UV lamp at the distance of 50 cm. After irradiation the conidial suspension were incubated at 20°C for 60 min in dark. After incubation, 0.1 ml was poured on solidified PMP medium to restrict the growth of fungal colonies. The plates were incubated at 20°C for six days until the fungal colonies were observed. Those isolates which retained the altered characters were identified as mutant.

### Screening improved strains

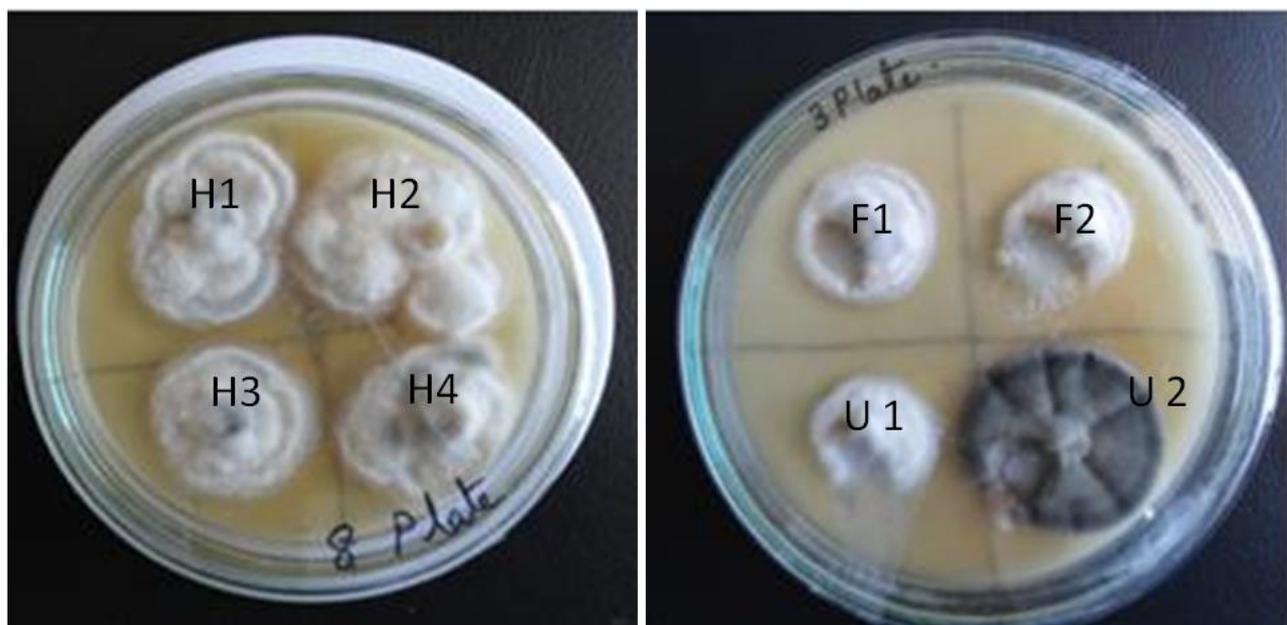
The strains were screened for biomass, exo-polymer and cordycepin production; all strains were inoculated in PMP pH6.0 and analyzed the production of mycelial biomass and exo-biopolymer, strains that produced maximum biomass and exo-biopolymer were selected for the further studies.

Disc measuring 1cm diameter of selected cultures were inoculated into PMP mediums and inoculated at 20°C at pH 6.0 for two weeks with initial four days under shaking. After cultivation in liquid PMP, the mycelial biomass was harvested by pelleting the culture at 10,000 rpm for 10 min. Dry weight of mycelia was recorded. The exo-polymer was extracted by concentrating the supernatant to 1/4th of its original volume and mixed with four times with absolute ethanol, stirred vigorously and then incubated overnight at 4°C for precipitation (Bae et al. 2000). The cordycepin was extracted from the liquid phase by extracting sequentially from hexane, chloroform and butanol (1:10 w/v for all solvents) the butanol extract was further purified in silica gel column with chloroform: methanol in 9:1 ratio. Cordycepin was analyzed by HPLC according to Wen et al. (2014b).

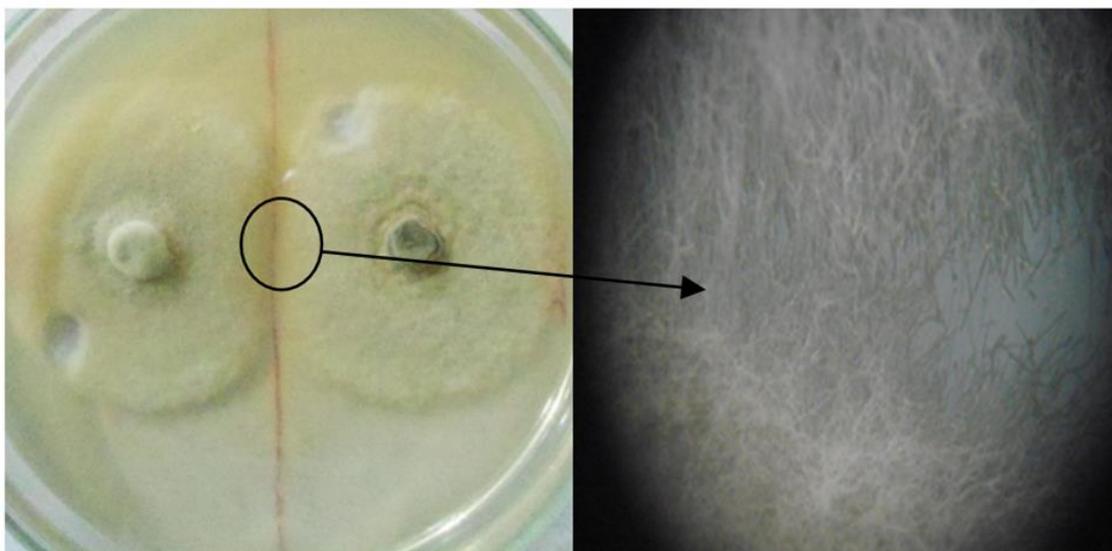
### Result

#### Hybridization

The growth of both strains MTCC-3936 and CCM-1 were inhibited by abrin for 3 days at the furrow region and after 3 days the mycelia started growing but the mycelia growth was slow. It was observed that at the meeting point the stroma was producing (Fig.1) and was able to grow faster than earlier. The 10 stromas were isolated from the meeting points into fresh CMA plates and inoculated at 20°C for two weeks. After incubation, disc of 1 cm from each plates were inoculated into PMP medium for evaluating the biomass and exo-polymer production. Four hybrid strains H-1, H-2, H-3 and H-4 (Fig. 2) were selected for future studies based on the high biomass production. To best of the knowledge the use of abrin to trigger somatic fusion is not yet reported. The study indicated that abrin is also an effective trigger to develop hybrids. The present study showed that *C. militaris* can withstand the inhibitory properties of abrin after somatic fusion and thus can be used as a good mode for effective hybridization since it inhibits protein synthesis of cells. The hybrids produced through this method have stability in biomass and exo-polymer production even after repeated sub culturing even after year storage (Data not given).



**Fig. 1**– Strains of *C. militaris* (MTCC-3936 (A) and CCM-1(B) in corn meal agar plate showing a growth inhibition in the presence of abrin and stroma development



**Fig. 2** – Improved *C. militaris* strains through hybridization (H1, H2, H3 and H4), Protoplast fusion (F1 and F2) and UV-irradiation (U1 and U2)

### Protoplast fusion

The re-suspended spores in protoplasting buffer were investigated for clumps under microscope. After fusion white colored strains of combination were observed were selected for further studies. Two fused cultures (F-1 and F-2, Fig. 2) were selected for comparative analysis based on high biomass production. It was observed that the strain were stable after sub culturing with similar biomass production rather decreased metabolites. It was also observed the strain were having combined characteristics both parents. The present study inactivation procedure using heat and UV was not followed to stabilize the fusion rather the fused suspension was directly inoculated to CMA from which the culture was isolated. It was observed that the isolates were stable even after sub-culturing.

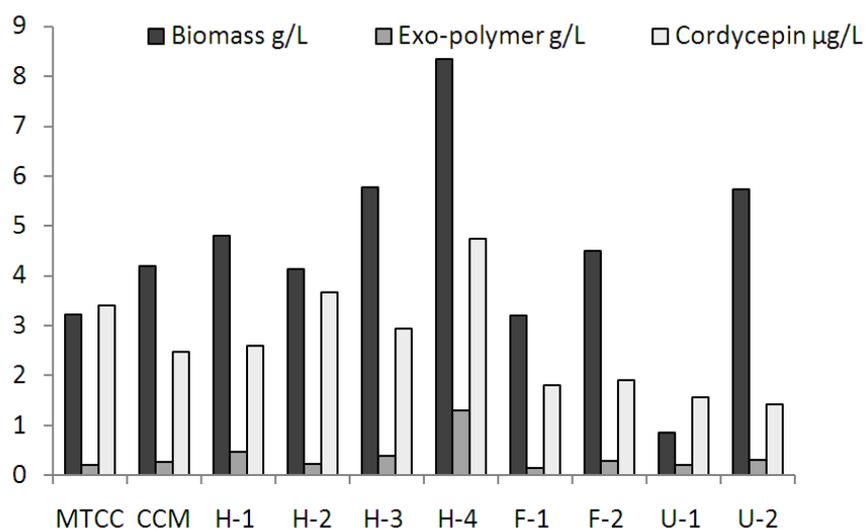
### UV-irradiation

After incubated for six to seven days, only an average of five colonies were observed viable and it was inoculated in 150ml of PMP broth and incubated for 10 days at 20°C. After incubation the culture was grown in CMA plates (Fig 2). U-1 (MTCC-3936), U-2 (CCM-1) from each parents were selected for further analysis. It was observed that the U-2 isolate showed change in it colony color from white to black colony (Fig. 2). More over the U-1 was having slow growth indicated mutation. It was observed that by this method the harvest of live spores and the viability of the cells were less. It was also observed that after repeated sub culturing the mutant colony were losing its characteristics and viability.

### Characterization of Hybrids

There was a higher biomass production in the hybrids as considered parental culture (MTCC-3936 and CCM) rather the cultured developed through plasma fusion where having similar parental characteristics. It was observed that the UV treated culture had morphological differences; U1 mutant had less mycelia biomass (0.86 g/L, Dry weight). Moreover the U-2 mutant developed from CCM culture had triggered mycelia growth than the parental strain. The three methods were effective in triggering the metabolic production and mycelia growth (Fig. 3). It was observed that, the hybrid H-4 developed by somatic fusion with abrin had double the biomass, exo-polymer and cordycepin production as compared to both parental strains. In comparison the developed strains showed higher biomass, exo-polymer and cordycepin production (Fig. 3) rather the plasma fusion was not triggering biomass as well as metabolic production. The present study indicated that abrin is capable to develop

strains with high metabolic products and biomass and will be a promising method for strain development in filamentous fungi.



**Fig. 3** – The biomass, exo-polymer and cordycepin produced by the improved cultures of *C. militaris*

### Discussion

Shrestha et al. (2005) mated two strains of *C. militaris* in CMA medium and showed the potential improvement in metabolic production. The present study is based on the study conducted by Holliday et al. (2004) using Snake venom for somatic fusion of *C. sinensis* and the developed strains were reproducible strain and showed improved quantitative bioactive compounds. A similar effective somatic fusion was observed in the present study using abrin, a plant poison. The antifungal property of abrin isolated from *A. precatorius* has been reported earlier (Prashith et al. 2010, Roy et al. 2012, Bhatia et al. 2013). It was observed that abrin can inhibit the growth of *C. militaris*, rather after somatic fusion the strain the effect of abrin. It can be stated that *C. militaris* had somatic fusion due to the inhibitory property of abrin and future the fused colony survived the effect of abrin in the long time exposure.

The present study supports the approach conducted by Zhou & Bian (2007) which showed increased cordycepin production and genetic stability in plasma fused strains of *C. militaris*. Moreover Guo et al. (2010) successfully fused *C. sinensis* and *C. militaris* and showed morphological and genetically similar to the parent strains. Moreover in both studies of plasma fusion heat and ultraviolet inactivation was done to stabilize the fusion but in the present study inactivation procedure was not followed to stabilize the fusion rather the fused suspension was directly inoculated to CMA from which the culture was isolated. It was observed that the isolated were stable even after sub-culturing and the colonies were having similar parental characteristics.

The use of low-energy ion beam in *C. militaris* can help to increase cordycepin production (Li et al. 2009). Das et al. (2008) demonstrated that the proton beam irradiation increases the production of cordycepin and it was observed that the mutant had a superior production performance. The present study showed that UV irradiation can be used for strain development; rather this process produces mutants which should be completely evaluated as a new strain. Based on the study of Holliday et al. (2004) showcased the effectiveness of the somatic fusion using snake venom in strain improvement, this study showed the effectiveness of abrin as a somatic fusion trigger.

### Conclusion

The three techniques employed for strain alteration, were found to be effective in *C. militaris*. The hybrids produced were stable and showed improved the strains productivity. The present study shows that, the somatic fusion is more effective than plasma fusion. Moreover UV irradiation is a

method of development of mutants and cannot be applied as an effective method. The protoplast fused strains were having similar parental characteristics. This study showed that the three techniques are effective in developing improved strain of *C. militaris* and can be further utilized to develop effective strains for metabolite and biomass production. The UV method had produced characteristic changes which were not stable and had produced mutants. The both fusion methods developed hybrids and were effective in producing quantitative higher biomass and metabolites. Since somatic fusion is an effective approach in strain alteration and abrin is a plant compound which can be easily extracted and are equally effective in fusion, makes it as an effective method of strain improvements in *C. militaris*. Moreover this approach can be evaluated in other fungal species.

### Acknowledgement

This project was funded by Sarvajanic Jankalyan Parmarthik Nyas, Bhopal. We are grateful to Mr S.N. Vijaywargia, Chancellor of People's University and Ms Megha Vijawarghia, Director HR & IT, People's University, for extending all the facilities for carrying out this study in Centre for Scientific Research and Development, People's University, Bhopal, India.

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