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Studies on the biology of *Cordyceps militaris*: A medicinal mushroom from North West Himalaya

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ABSTRACT

Cordyceps militaris (L.) Link is an entomopathogenic fungus, which enjoyed an extensive medicinal utility. In this study, taxonomic details, isolation of pure culture, influence of different physiological requirements on the mycelial growth of this fungus, its chemical components and molecular characterization has been carried out. The present investigation revealed that this fungus showed optimum growth in YPDA (yeast potato dextrose agar) and GAS (glucose asparagine solution). The maximum mycelial growth was observed at 25 °C and pH 7.5 and 5.5 in solid and liquid media, respectively. Among various carbon, nitrogen, mineral and vitamin sources tested, sucrose, beef extract, zinc chloride and folic acid produced the maximum mycelial yield, respectively. The fungus was found rich in various chemical components like vitamins, proteins trace elements, cordycepin and cordycepic acid. *C. militaris* has been successfully cultivated under lab scale cultivation trials under standardized nutritional and climatic conditions.

Keywords: *Cordyceps militaris*, medicinal mushroom, entomopathogenic fungus, artificial cultivation, nutritional components.

INTRODUCTION

Medicinal fungi have long been an important part of human civilization, and species of the genus *Cordyceps* are especially valued (McKenna *et al.*, 2002). *Cordyceps*, a macrofungus that is parasitic on insects, is used as a source for development of functional food and new drug discovery (Xiao *et al.*, 2009). It belongs to phylum *Ascomycota*, class *Ascomycetes*, order *Hypocreales* and family *Clavicipitaceae* (Holliday and Cleaver, 2008). While not actually a mushroom in the taxonomic sense, it has been regarded as, a medicinal mushroom throughout history. The name comes from the Latin words: *cord* and *ceps*, meaning “club” and “head”, respectively. The Latin conjugation describes the appearance of the club fungus, *Cordyceps sinensis*, whose stroma or fruitbody extends from the mummified carcasses of insect larvae, usually caterpillar larva of the Himalayan bat moth, *Hepialis armoricanus*. *Cordyceps* mushroom has a long history as medicinal fungus. It has been regarded as a cornerstone of traditional Chinese medicine for centuries; that apparently have a number of far reaching medicinal effects (Mizuno, 1999; Holliday *et al.*, 2005). *Cordyceps* mushrooms have been used to treat conditions including respiration and pulmonary diseases, renal, liver, cardiovascular diseases, hyposexuality and hyperlipidemia. It is also used in the treatment of immune disorders, and as an adjunct to modern cancer therapies (Holliday and Cleaver, 2008; Khan *et al.*, 2010).

MATERIALS AND METHODS

Survey, collection, taxonomic studies and isolation of pure culture

Fruiting bodies of *Cordyceps militaris* (L.) Link have been collected from Glen forest and Tara Devi (Distt. Shimla, H.P.) and its adjoining areas falling in the overall geographic limits of North West Himalaya during the months of June to September. Fruiting bodies have been preserved dry as well as wet (Ainsworth, 1971) and the specimens have been deposited in the Herbarium of Department of Biosciences, Himachal Pradesh University,

Shimla.

Macroscopic studies

Various characters, which help in the identification of specimens e.g. shape, size and colour of the stipe and stroma, association with insect larvae and pupae were recorded by examining the specimens with naked eye. The specimens were identified by following Lincoff (1981).

Microscopic studies

For microscopic studies both dried as well as wet preserved specimens were used. The dried parts of specimen were kept for few minutes in 95% ethyl alcohol (to expel out the air) and then in water. The anatomical details of the specimen was worked out by cutting free hand sections of the material. Microscopic details of the specimen was worked out in laboratory with the help of research microscope. This included the study of mycelium and spores. For clarity the sections were stained with 1% cotton blue and lactophenol. The sections were observed under the microscope. Photomicrographs of slides of mycelium and spores were taken and measurements were recorded with micrometer.

SEM Studies

Surface of mycelium and spores were imaged with the help of Scanning Electron Microscope (SEM). For scanning electron microscopy, samples were mounted on carbon tape and were placed on the stub, then placed in Environmental Scanning Electron Microscope Mode (ESEM MODE) under vacuum and desired pressure, the images of the samples were obtained on screen.

Isolation of pure culture of *Cordyceps militaris*

The cultures were raised from the stipe and stroma portion of healthy, sun-dried and fresh specimens. The specimens were first washed with distilled water and then the tissue from the stipe and stroma portion were cut with the help of a sterilized blade. The bits of tissue (2-3 mm) were taken up with a sterilized forceps and dipped in 0.1% mercuric chloride solution for 5-10 seconds. Now the tissue was

placed on filter paper to remove the excess moisture. The small bits of *Cordyceps* tissues were then transferred aseptically into the petriplates containing potato-dextrose agar (PDA) medium with the help of a sterilized forceps. These were then incubated at 25 °C for at least 8-10 days and observed regularly for appearance of culture. The actively growing mycelial colonies were sub cultured to obtain pure cultures.

Physiological Studies

Twelve solid and five liquid media have been tried during the present studies. All media were prepared following (Tuite, 1969).

Inoculum preparation

Mycelial discs of 5 mm diameter were taken out with a pre-sterilized borer under aseptic conditions, to be used as inoculum in solid media. For liquid media, the mycelial disc of 5 mm was transferred to 250 ml flask containing 50 ml of liquid medium and incubated for 25 °C for 8-10 days. After 10 days, there appeared a ball of mycelium, which was homogenized in the medium by sterilized rod. The 5 ml of this homogenized mycelium was added to each of different liquid media as an inoculum used for further studies.

Recording of vegetative growth in solid and liquid media

Vegetative growth of mycelium in the solid media was measured by taking the diameter of colony in two directions at right angles. In liquid media, the mycelial mats were filtered through Whatman filter paper No.1 and weighed to give dry weight of the mycelium. The medium with best mycelial growth was used for further studies.

Effect of temperature

For the study of temperature requirement of the fungus in solid and liquid media, inoculated petriplates and flasks were incubated at different temperatures viz. 5, 10, 15, 20, 25, 30, 35 and 40 °C in separate incubators on the best suited solid and liquid media.

Effect of hydrogen ion concentration (pH)

To record the effect of different pH on the growth of this fungus, the best solid and liquid media, were adjusted at different pH levels, ranging from 4.0 to 10.0 with the help of NaOH and HCl which was checked by making use of digital type Philips pH meter. The inoculated petriplates and flasks were incubated for 10 days at optimum temperature and after that the growth was measured.

Effect of light and darkness

Best selected solid and liquid media with optimum pH after inoculation was given light and dark treatment at optimum temperature. Growth was observed after 10 days of incubation.

Effect of different carbon, nitrogen, minerals and vitamin sources

To find out the best carbon, nitrogen, mineral and vitamin sources for the growth of fungus, the best liquid basal medium was substituted by different nutritional sources. Mycelial weight on dry weight basis was recorded after 10 days of incubation.

Determination of different chemical components

The various nutritive and chemical components like proteins, vitamins, trace elements, cordycepin, cordycepic acid, polysaccharides and superoxide dismutase were determined following standard techniques and methods (Raghuramulu *et al.*, 2003).

Artificial cultivation trials

Mass inoculum was prepared on wheat grains following the method of Stoller (1962). Lab scale cultivation trials were performed on grains and potato broth medium supplemented with different nutrients and subjected to different conditions (Dark, Light and in BOD) in laboratory.

RESULTS

Macroscopic characters

The fruiting body of *C. militaris*, appeared creamish white in colour and showed intimate association with insect larvae and pupae. The associated insect's body becomes mummified by the growth of the mycelium. The mycelium of the fungus forms fruiting bodies, which interestingly, always emerge from the head of the larva. The size of fruiting body varied from 4.2 to 7.8cm (Plate 1, a-b).

Microscopic characters

Conidiophores sub cylindrical, 210.2-212.4 x 45.3-46.3 µm; phialide flask Shaped, 223.3-226.1 x 48.7-49.8 µm; conidia barrel Shaped, 217.2-223.2 x 47.6-49.9 µm;

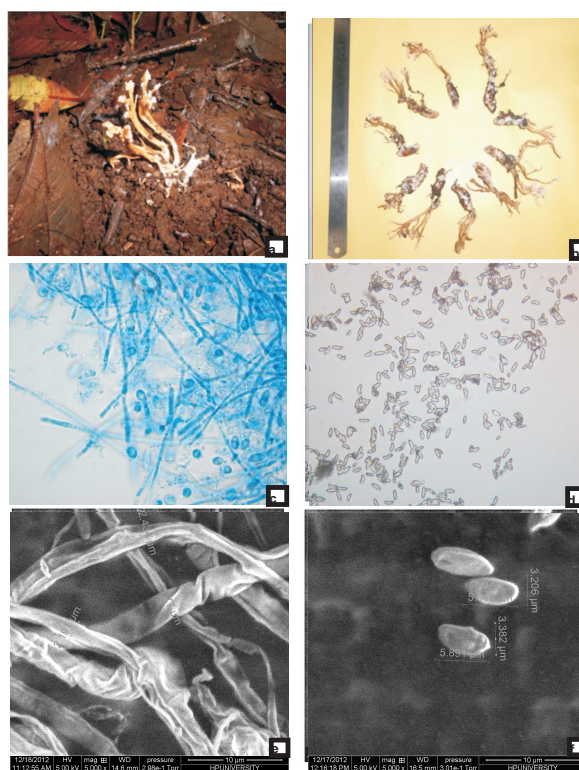


Plate 1: a) *Cordyceps militaris* in its natural habitat. b) Fruiting bodies of *C. Militaris* in association with insect host. c) Microphotograph of mycelium of *C. militaris*. d) Microphotograph of spores of *C. militaris*. e) SEM images of hyphae of *C. militaris*. f) SEM images of spores of *C. Militaris*.

Table 1: Showing some nutritional and nutraceutical constituents

Protein (%)	Vitamins (mg/g)					Trace elements (ppm)			Cordycepin (%)	Cordycepic acid (%)	Polysaccharides (%)	SOD (Units/mgprotein)
	A	B ₁	B ₆	B ₁₂	B ₃	Se	Zn	Cu				
39.15	32.7	12.4	58.7	69.3	40.6	0.35	127.1	27.7	1.24	10.79	28	51

hyphae thin walled, branched, 14.8-16.1 μm broad (**Plate 1, c-d**).

SEM Studies

The mycelia and spores of *C. militaris* were observed under Scanning Electron Microscope (SEM) at different magnification at pressure 2.9e-1 Torr. The diameter of hyphae generally ranged from 3.18 μm to 2.49 μm . Spore surface of fungus was smooth and velvety, its size ranged from 5.82-5.89 x 3.20-3.82 μm (**Plate 1, e-f**).

Mycelial characteristics

Mycelial growth of *C. militaris* was longitudinally radial, aerial initially, creamish white, becoming densely matted and wooly in texture. As soon as the colony matures the colour of mycelium changed from creamish white to light brown. As the medium was completely consumed, the mycelia became increasingly mud-like and granular in texture. At approximately 16 days of growth or a bit later, the mycelium of *C. militaris* began to form small nodules (perhaps sclerotia) at the centre on the surface of the medium, appearing light brown while peripheral mycelia

remained creamish white (**Plate 2, a-b**).

Physiological Studies

Among the twelve solid and five liquid media tried, Yeastal Potato Dextrose Agar and Glucose asparagine solution were found to be the best solid and liquid media respectively (**Plate 2, b-c and Figs. 1-2**). The best mycelial growth of *C. militaris* was observed at 25 °C both in solid and liquid media (**Figs. 3-4**). The best mycelial growth of *C. militaris* was observed at pH 7.5 and 5.5 in solid and liquid media respectively (**Figs. 5-6**). With regard to the effect of light and darkness on solid medium, the mycelium was found to give better growth under darkness in comparison with light (**Figs. 7**). Among the five carbon, six nitrogen, six mineral and six vitamin sources tested, sucrose, beef extract, zinc chloride and folic acid produced the maximum mycelial yield, respectively (**Figs. 8-11**).

Chemical components of *C. Militaris*

Fruiting bodies of *C. militaris* have been found to be rich in different biochemical components (**Table 1**)

Mass inoculum and lab scale cultivation trials

Mass inoculum was prepared on different substrates (wheat grains, maize grains and sorghum) following standard method as described in methodology for lab scale cultivation trials of *C. militaris* on different substrates (**Plate 2, d-f**). Polypropylene bags containing sterilized wheat grains and sorghum were inoculated with pure culture of *C. militaris*. These bags were incubated at 25 °C for 15 days in a dark room. Entire grain surface was colonized by the fine thread like mycelium of fungus. These polypropylene bags were then opened and subjected to different treatments like moisture, humidity, temperature and light variations. It was noticed that bags subjected to moderate day light (2-4 hours), optimum temperature (25 °C), 50%-70% atmospheric humidity started showing vertical stromata which resulted in the formation of complete fruiting bodies in 50-60 days (**Plate 2, g-i**).

DISCUSSION

During present study specimens of *C. militaris* collected from different sites of Shimla district of Himachal Pradesh were investigated for their macroscopic and microscopic details which are almost in conformity with the observations of Sehgal and Sagar (2006). SEM study on *C. militaris* has been conducted for the first time in India.

Among the twelve solid and five liquid media tried, yeastal potato dextrose agar and glucose asparagine solution were

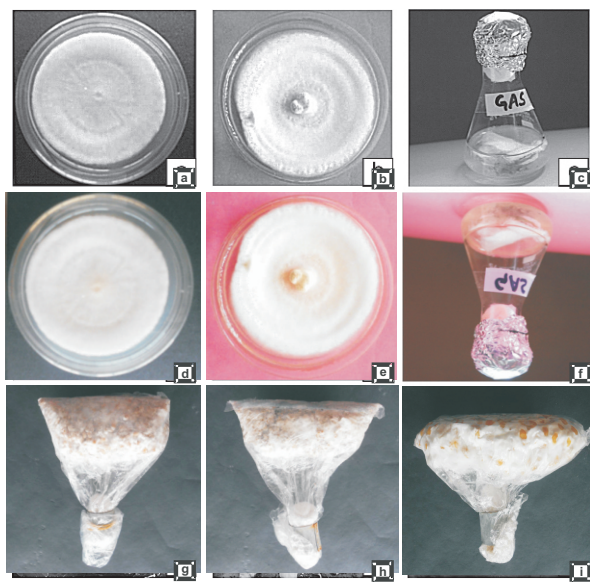


Plate 2: a) Pure culture of *Cordyceps militaris*. c) Growth of *C. militaris* on Yeastal Potato Dextrose Agar. c) Growth of *C. militaris* on Glucose Asparagine Solution. d) Mass inoculation of Wheat grains. e) Mass inoculation of Sorghum grains. f) Mass inoculation of Maize grains. g) Artificially cultivated fruiting bodies of *C. militaris* on Wheat grains. h) Artificially cultivated fruiting bodies of *C. militaris* on Maize grains. i) Artificially cultivated fruiting bodies of *C. militaris* on Broth Medium.

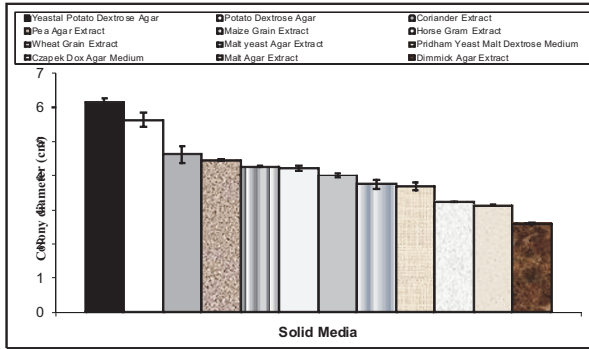


Fig. 1: Effect of different solid media on the growth of *C. Militaris*

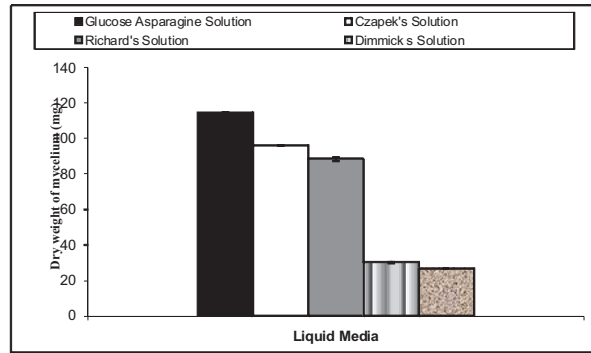


Fig. 2: Effect of different liquid media on the growth of *C. Militaris*

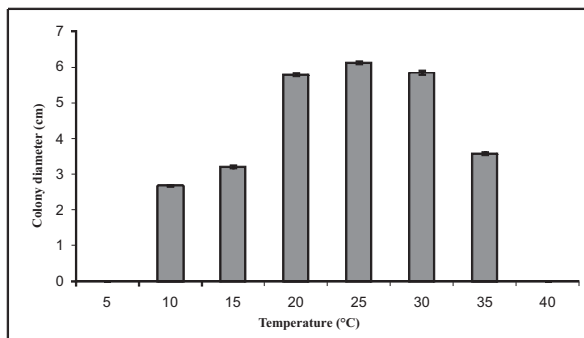


Fig. 3: Effect of temperature on the growth of *C. militaris* in basal solid medium

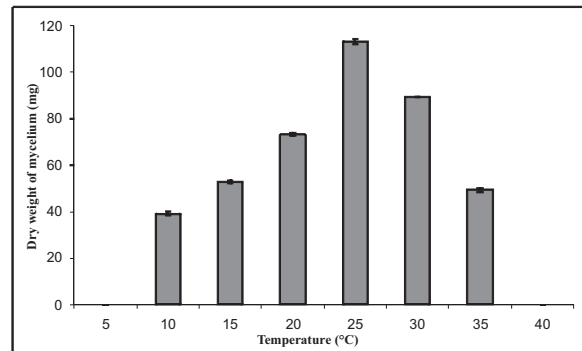


Fig. 4: Effect of temperature on the growth of *C. militaris* in basal liquid medium

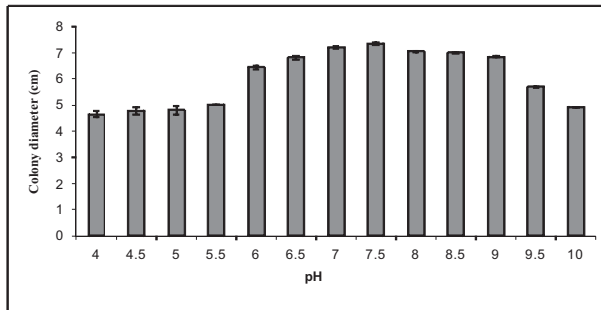


Fig. 5: Effect of different pH levels on the growth of *C. militaris* in basal solid medium

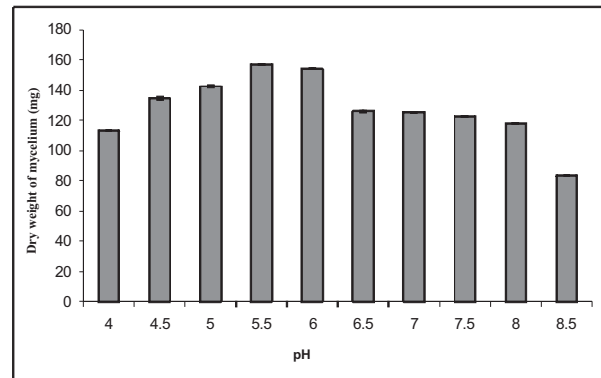


Fig. 6: Effect of different pH levels on the growth of *C. militaris* in basal liquid medium

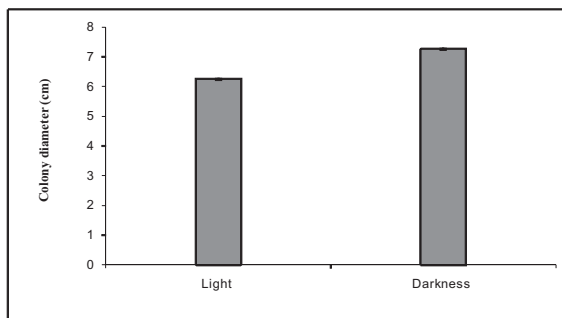


Fig. 7: Effect of light and darkness on the growth of *C. militaris* in basal solid medium

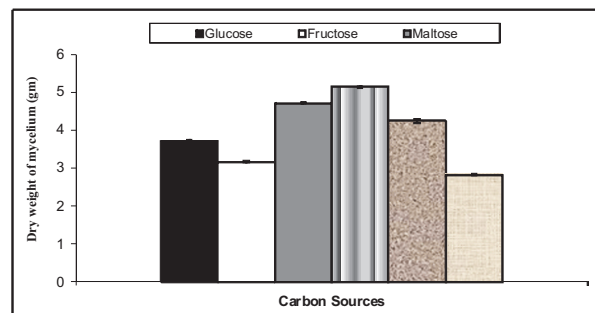


Fig. 8: Effect of different carbon sources on the growth of *C. militaris* in basal liquid medium

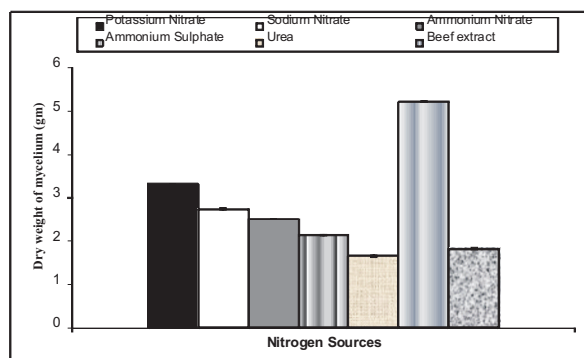


Fig. 9: Effect of different nitrogen sources on the growth of *C. militaris* in basal liquid medium

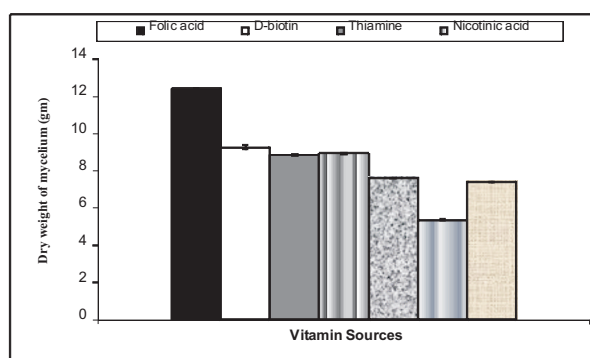


Fig. 10: Effect of different vitamin sources on the growth of *C. Militaris* in basal liquid medium

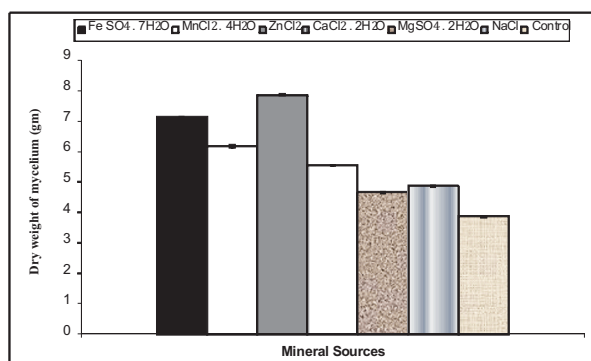


Fig. 11: Effect of different mineral sources on the growth of *C. militaris* in basal liquid medium

found to be best solid and liquid media at 25 °C, respectively. The best mycelial growth of *C. militaris* was observed at pH 7.5 and 5.5 in solid and liquid media, respectively. With regard to light and darkness, the mycelium was found to show better growth under darkness. In case of different carbon, nitrogen, minerals and vitamins tried, sucrose, beef extract, zinc chloride and folic acid produced the maximum mycelial yield respectively. All results are in agreement with earlier reports with minor variations which can be attributed to strain specific behavior of the fungus.

Present investigations confirmed the presence of carbohydrates, proteins, fats and different minerals in the fruiting bodies of *C. militaris* collected from Himachal Pradesh. There is a need to evaluate this mushroom for carbohydrates, amino acids, minerals and vitamins.

C. militaris has been artificially grown successfully under lab scale cultivation trials on wheat and maize grains substrates. There are few reports (Wang *et al.*, 1992; Hong *et al.*, 2010) in literature which claim artificial cultivation of *C. militaris* using different substrates including insect dead bodies. But none of such investigations provides a clear methodology for commercial cultivation of this mushroom.

In the Indian mushroom scenario, this is the first report of successful artificial cultivation of this medicinal mushroom. Nearly 80-85% of all medicinal mushroom products are extracted from their fruiting bodies while only 15 % are derived from mycelium culture (Lindequist *et al.*, 2005). As all physical and chemical requirements for the growth of *C. militaris* have been standardized in the

present investigation, there is a scope to extend this work for commercial cultivation of *C. militaris* using agro-wastes which are available in plenty in India.

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