

# ARTIFICIAL CULTIVATION OF HIGH VALUED MEDICINAL FUNGUS *OPHIOCORDYCEPS SINENSIS* ON IN-HOUSE DEVELOPED VEGETARIAN MEDIUM

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**Abstract**—*Ophiocordyceps sinensis*, or caterpillar fungus, is a highly valued entomopathogenic fungus exclusively from the Himalayas, prized for its health benefits. As natural populations decline due to increased demand, there is growing interest in cultivating it in labs. However, lab-grown varieties often rely on non-vegetarian mediums, limiting their appeal to vegetarians and vegans, and quality concerns also persist. In this study, natural forms of *O. sinensis* were collected from North Sikkim, India, at an altitude of 17,500 feet. The mycelia were cultured on an all-vegetarian medium using holy millet *Echinochloa frumentacea* (*Bhagar* in Hindi). Culture attained maturity in 12 months. The presence of cordycepin was established using HPLC-UV which confirmed the authenticity of the lab-grown fungus and demonstrating its potential for commercial cultivation.

## INTRODUCTION

*Ophiocordyceps sinensis*, formerly known as *Cordyceps sinensis*, is highly esteemed for its potential to boost energy levels, enhance longevity, and improve overall quality of life (Zhu, *et al.*, 1998; Yao, *et al.*, 2024). This Ascomycetes fungus primarily targets the caterpillar of the moth species *Hepialis armoricanis*, using its mycelia to parasitize the host and eventually forming a stroma or fruiting body. The characteristic club-shaped mycelia represent the fungus's reproductive structure, while the caterpillar constitutes its host. Revered for centuries, *Ophiocordyceps sinensis* is considered a rare and exotic medicinal fungus, playing a foundational role in traditional Chinese medicine (Holliday and Cleaver, 2008).

*Ophiocordyceps sinensis* is rich in a variety of bioactive compounds, including both macro and micro molecules. Among its most significant components are cordycepin (-3'-deoxyadenosine) (Tuli, *et al.*, 2014) and cordycepic acid, which are recognized for their potent effects (Zhou, *et al.*, 2009). This fungus also contains essential amino acids, vitamins such as A, E, K, B1, B2, and B12, as well as carbohydrates, proteins, sterols, nucleosides,

and vital minerals like magnesium, iron, copper, manganese, zinc, phosphorus, selenium, aluminum, silicon, nickel, strontium, titanium, chromium, potassium, sodium, and calcium.

As a result of overharvesting wild populations, there has been a dramatic increase in the demand for *O. sinensis*. Consequently, most of the *O. sinensis* available in the market today is cultivated artificially. The artificial culturing of *O. sinensis* has been performed by different methods, such as using the host insect, microbiological medium containing animal extracts such as peptone and beef extracts (Sharma, *et al.*, 2024). Although, many of these reports have shown that these artificially cultured products carry almost same biochemical profiles, as of natural form of *O. sinensis*, such products may not be useful in the Indian context, as majority being vegetarians, may not accept such products grown on insects, or with animal-based extracts *i.e.*, beef extract, meat extract. Hence, our idea was to introduce a completely vegetarian medium, that can be used to artificially culture the *O. sinensis*, and can be used without any further purification needs.

Several reports have been shown that many substandard products are marketed under the guise of pure *O. sinensis*. making it essential for

distinguishing genuine products from inferior imitations. The present study was successful in cultivating the *O. sinensis*, from its natural form collected from North Sikkim, and to establish its authenticity using its signature compound, cordycepin.

## MATERIALS AND METHODS

### Sample collection

For artificially cultivation, the natural forms of *O. sinensis* were collected from its natural habitat; from high altitude areas (~ 17500 ft. from mean sea level) in North Sikkim, India (Fig. 1). The samples were carefully packed and brought to the laboratory under refrigerated condition. In laboratory, these natural forms of the fungus were washed several times with sterilized water carefully. The mycelia were collected by dissecting the outer body of the fungus. These mycelia were used as a starting culture for artificial cultivation.



Fig. 1. Natural form of *Ophiocordyceps sinensis*

### Establishment of initial culture

The mycelia obtained from the natural form of *O. sinensis* were inoculated on potato dextrose agar medium and the plates were incubated at  $18 \pm 1$  °C in a BOD incubator, till the mycelia grew on the plates. Once the mycelia are grown on potato dextrose agar, the *in vitro* cultured mycelia were transferred to the liquid medium on potato dextrose broth.

### Development of in-house culture medium

For the growth and maintenance of the *O. sinensis* mycelia, we developed a completely vegetarian medium. For 1 kg semi solid media, 15 g barnyard millet (*Echinochloa frumentacea* also known as *Bhagar* in Hindi) was mixed with 10 g dextrose and 5 g  $\text{CaCO}_3$  in 1 L of water. When, the medium was autoclaved at 121 °C and 15 psi pressure for 15 min, a semisolid culture medium was obtained, which was used for mass cultivation of *O. sinensis* starting culture.

### Mass cultivation of *O. sinensis*

The starter liquid culture was transferred to the in-house developed, sterile vegetarian medium in a 250 ml tissue culture bottle and capped. The bottles were incubated  $18 \pm 1$  °C in a BOD incubator under dark for several weeks, until all the media is consumed by the mycelia.

### Authentication of artificial culture

Once, the mycelia growth was found sufficient, the whole content of the bottle was lyophilized to get the powder form. Lyophilized uninoculated medium, incubated under the same conditions for the same time served as negative control.

Cordycepin was extracted from the lyophilized crude material was extracted with 50% ethanol in water (v/v) under constant stirring at 150 RPM for 10 h. The resultant mixture was centrifuged at 10000 g and the supernatant was subjected to solid phase extraction using Envi C-18 cartridges (500 mg, Merck, Germany). The bound material was eluted with 3 ml of methanol.

Further quantification of cordycepin was done using High Performance Liquid Chromatography with ultra violet detection (Choi, *et al.*, 2021). Cordycepin pure compound (Sigma Aldrich, USA) was used as a standard, dissolved in methanol in  $10 \mu\text{g ml}^{-1}$  concentration. Sample or cordycepin standard ( $10 \mu\text{l}$ ) was injected via SIL autosampler (Shimadzu, Japan) to HPLC column (C18, 250 mm x 5 mm, 5  $\mu\text{M}$  pore size) using a binary gradient program. Mobile phase A was 0.5% acetic acid in water and mobile phase B was acetonitrile. The gradient program was as follows: 100% A at 0 min, 15% A at 3 min, 0% A at 15 min, 100% A at 25 min. The run was at 30 °C and the detection was done at 261 nm using an ultraviolet detector (Shimadzu, Japan).

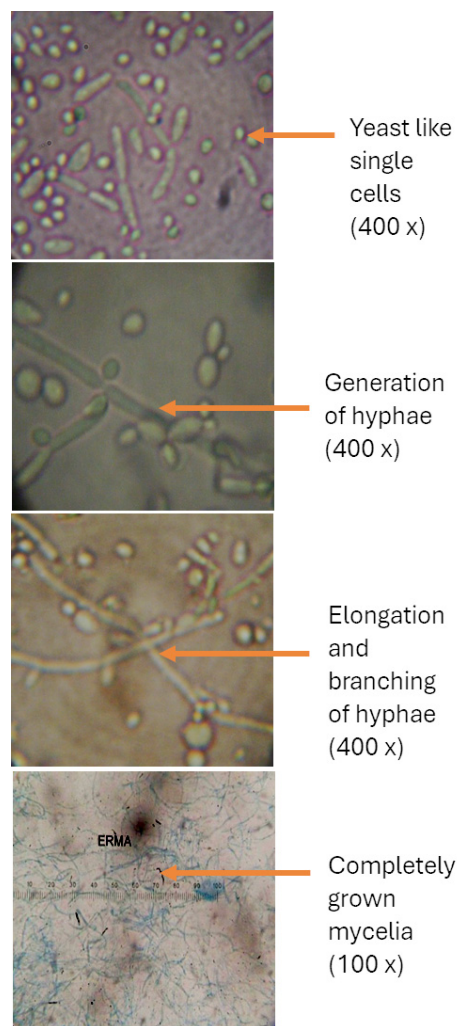
## RESULTS

The initial culture of *O. sinensis* grew as white to creamish colored colony on potato dextrose agar with a radial growth of 15 mm in 60 days. The growth on potato dextrose agar was transferred to potato dextrose broth. The culture attained maturity in liquid broth in 30 days, showing thin mycelia. Further, the liquid broth, containing culture, was transferred to in-house developed vegetarian medium, and allowed to grow up to 12 months (Fig. 2).



**Fig. 2.** Establishment of mycelial culture of *Ophiocordyceps sinensis* on vegetarian medium

For the first month, the fungus grew initially as single celled yeast-like colonies, which slowly enlarged to hyphae. As visualized under the microscope (400 x magnification), the single cells elongated and make hyphae, which slowly converted into mycelium (Fig. 3). The culture attained maturity in 12 months, characterized by the complete exhaustion of medium by the fungus. At

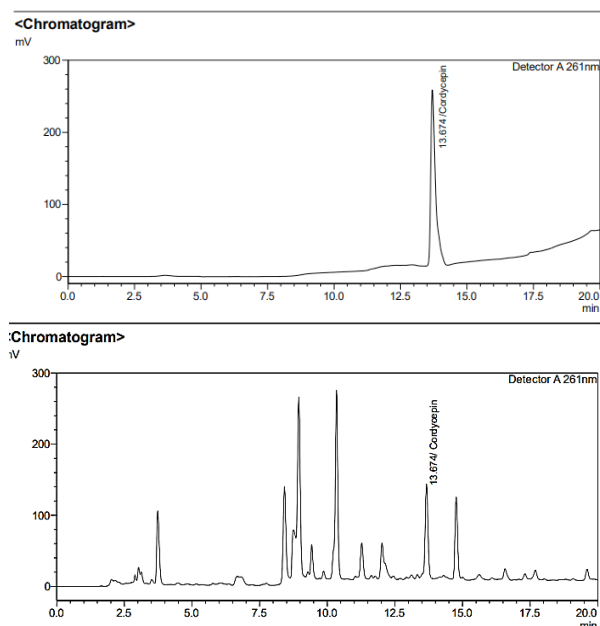


**Fig. 3.** Microscopic images of different growth stages of *Ophiocordyceps sinensis* under vegetarian media

maturity, single cells were almost nil, while culture could be seen as a lump of thin mycelia.

The culture after attaining maturity was lyophilized to get the powder, and extracted with ethanol. The cordycepin was extracted and concentrated using solid phase extraction technique, finally dissolved in 3 ml of methanol and subjected to RP-HPLC with UV detection. In order to identify the cordycepin in the laboratory grown culture, the chromatogram was matched with that of pure cordycepin in  $10 \mu\text{g ml}^{-1}$  concentration, run under same conditions.

Fig 4 shows the single peak of standard cordycepin at 13.674 min. The purified extract of lab grown culture revealed many peaks. Cordycepin was identified with a peak at same retention time as of standard (13.674 min). further confirmation was obtained by spiking the culture extract with standard cordycepin resulting in enlargement of the peak at 13.674 min. The uninoculated media, extracted in the same way did not show any peak in the chromatogram.



**Fig. 4.** High performance liquid Chromatogram of A: standard cordycepin, and B: *in vitro* grown culture of *Ophiocordyceps sinensis*. Cordycepin presence was established at RT 13.674 min, as compared to the standard cordycepin, which eluted at same RT.

## DISCUSSION

*Ophiocordyceps sinensis* is a rare and high value medicinal fungus (Shrestha, *et al.*, 2010), and many

attempts are being made for its artificial culturing under in vitro conditions, keeping the spectrum of its bioactive metabolites intact. There has been significant research into the submerged cultivation of *Ophiocordyceps sinensis* (*O. sinensis*) mycelium over the years. As far as we dig into the literature, we found that all the artificial medium either contained extracts from animal origin or using the standard microbiological media that contained animal extracts such as beef extract (Lu, *et al.*, 2015). Our study is the first to develop the mycelial culture on a purely vegetarian media, enabling the vegetarians and vegans to use the artificial culture of *O. sinensis*.

Further, our culture media is semisolid and easy to handle in the laboratory, avoiding the large set up required for handling the liquid fermentation method (Dong and Yao, 2005). The cooked rice-like appearance provides enough space for mycelia to grow rapidly. After maturity, whole culture bottle can be harvested and used, as the medium left is very less, and non-toxic.

The culture was authenticated by the presence of the signature compound; cordycepin. Due to the vast DNA anomalies among anamorphs of nature grown as well as laboratory cultivated *O. sinensis*, which are reported to have similarities with more than 30 fungal strains (Zhou, *et al.*, 2014), authentication of *O. sinensis* has always been suspicious, and presence of bioactive signature compounds are more useful tools for such authentication.

It is well known fact that the natural forms of *O. sinensis* take approximately 5 years to attain maturity in the nature, which is one of the key factors responsible for scarcity in nature and high pricing. Since, the culture has been authenticated showing the presence of cordycepin, our culturing technique may provide an easy and cost-effective alternative for the *O. sinensis* to be used as health supplement.

This paper presents the primary investigation on culture authenticity, and further work on the optimization of culture conditions and presence of other important biochemicals is going on.

## CONCLUSION

Caterpillar fungus (*Ophiocordyceps sinensis*), is highly valued throughout the world as a dietary supplement or tonic food and natural remedy. The price of *O. sinensis* has continued to increase over the

last few years due to growing worldwide demand, driving research to determine methods of artificial cultivation to make *O. sinensis* a more affordable material for commercial trade. All the present culture methods use animal-based ingredients in culture media, causing hesitation amongst the vegetarians to use such product., We have developed a culture medium that is not only completely vegetarian, but uses the millet that is rated a holy food during the fasts in India.

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**Conflict of Interest-** None

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