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Growth media optimization strategies to achieve higher mycelial and fruiting body yield of *Cordyceps militaris* for large scale production

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ABSTRACT

Cordyceps militaris, grow in high altitude in Himalayan very harsh conditions it is big challenges for the researchers how to grown under control conditions, this study is based on optimization of maximum mycelial growth of *C. militaris.* and develop a standard protocol for high yield production of *C. militari.* The laboratory experiment was carried out to study various factors on the mycelium and fruiting body growth of *C. militaris.* A vigorous scientific study aimed to identify the various factors like nutrient media, temperature hydrogen ion concentration, and carbon source for the mycelium growth along with grain substrate and liquid medium for the high yield production of *C. militaris.* The findings revealed that the *C. militaris* was more likely to develop in CDA (Czapek Dox Agar) nutrient media and maximum mycelium growth were observed at the temperature of 20 °C whereas optimum growth occurred at pH 6. carbon sources such as glucose, and dextrose, at a concentration of 20g/l, enhanced the mycelium growth of *C. militaris*. further perform the study of fruiting body production in several grains, like brown rice, wheat, millet, chickpea, and maize and finding reveals that maximum fruiting body production found in brown rice along with performing study on different liquid media (M1, M2, M3, M4, and M5) with brown rice as a grain substrate, during observation and analysis conclude that the M3 liquid media producing the maximum yield and primordia and maximum fruiting body of *C. militaris*. This study parameter provides basic information for vegetative growth and high yield production protocol for *C. militaris.*

Key words : *Cordyceps militaris, Entomopathogenic, Nutrient media, Carbon source.*

Introduction

Cordyceps militaris is an Ascomycota phylum, Hypocreales order, and Clavicipitaceae family entomopathogenic fungus (Holliday and Cleaver, 2008). is widely used in traditional in South Asia as medicine as well as food. The Ascomycetes phylum, while it is not recognized as a mushroom by taxonomy, it has been traditionally used as a therapeutic fungus. The "Cordate Club" originated from the Latin words "cord" and "cephalic," which are related to "club" and "head," respectively. *Cordyceps* genus has been reported about 450 species include 110 species are found in South Asia (Kirk *et al*., 2001). *Cordyceps* is a well-known medicinal fungus that has been in use for thousands of years. It can be found on the skeletonized dead insect larvae of caterpillar larvae, typically Himalayan bat moth caterpillar larvae and Hepialis armoricanus. *Cordyceps* species are valuable fungi with therapeutic properties and have been widely used for centuries to ensure patient safety (McKenna *et al.,* 2002; Mehra *et al.,* 2017). *Cordyceps* is a macro fungus that is parasitic on insects, it is possible to produce functional

food and innovative therapeutic research (Xiao et al., 2009). For many years, it has been utilized in Asian medicine; study has shown that it has numerous health advantages (Mizuno, 1999; Holliday *et al.,* 2005). *Cordyceps* has been used to treat lung disease, renal disease, liver disease, cardiovascular disease, and excessive cholesterol. It is used to treat immunological disorders as well as chemotherapy. (Holliday and Cleaver, 2008; Khan *et al.,* 2010). Therefore, using modern scientific methodologies in the investigation of this species has shown anticancer, antioxidant, and immunomodulatory properties (Yoo *et al.,* 2004; Park *et al.,* 2005). *C. militaris.* mycelia and fruiting body production has been cultivated using advanced technology. Much research has been conducted to enhance cordycepin production in *C. militaris* (Masuda *et al.,* 2006). More recently, the different experimental methods applied in the growth of *C. militaris* fruiting bodies (Adnan *et al.,* 2017), have been improved to compensate for factors such as temperature humidity, and light intensity (Yang *et al.,* 2016; Lee *et al.,* 2013; Dang *et al.,* 2018).

The nutritional and therapeutic benefits of mushrooms have triggered the demand and cultivation of the fungus for food consumption (Fan *et al.,* 2006). Artificial cultivation improves the fruiting body and mycelial production of *C. militaris* many numerous experiments to improve Cordycepin production (Masuda *et al.,* 2006).

Methodology

The survey, collection, and preservation of culture

In the summer season, collections and isolation of the fruiting body of *Cordyceps militaris* were investigated in the high altitudes of Panchachuli (30° 12' 30.60" N and 80° 25' 23.39" E) and Kalamuni mountain (30.0377° N, 80.1998° E) in the town of Munsiyari, Pithoragarh, Uttarakhand. For isolation of the newly collected fruiting body of *C. militaris.* was cultured into PDA (Potato dextrose agar) media and incubate at 20±2°C for 7 days and culture was the store at 4°C for further use. (Rupesh Kumar Arora *et al.,* 2013).

Growth media optimization strategies for higher mycelial growth of *C. militaris.*

Effect of different nutrient media

Characteristics of the colony have been analyzed on Ten different media *viz.* YEMA (Yeast Extract Malt Agar), CDA (Czaeck Dox Agar), SCDA (Soybean Casein Dextrose Agar), MEA (Malt Extract Agar), YESA (Yeast Extract Starch Agar), MGYPA (Malt Extract Glucose yeast peptone Agar), PDA (Potato Dextrose Agar), SDA (Sabouraud Dextrose Agar), BEAD (Beef Extract Dextrose Agar) and CAN (Cordyceps Agar Nutrient). The inoculum, inoculated into media containing triplets, and this experiment was performed in triplicates, these Petri plates incubate at 20±2°C for 16 days, and radial growth was observed every 48 hours.

Effect of different temperatures

C. militaris cultures were inoculated into Petri plates with sterilized CDA (Czaeck Dox Agar) media under aseptic conditions and incubated for 10 days at 6 different temperatures *viz* 5, 10, 15, 20, 25, and 30°C. Three replicates were maintained for each treatment, and radial growth was observed every 48 hours.

Effect of different pH

For determining the effect of pH on CDA medium different pH levels *viz* 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10 of the medium were maintained before sterilization with the help of 1.0N NaOH or 1.0N HCL, then inoculated plates were incubated at 20±2°C for 10 days. each treatment was performed in triplicates and radial growth was observed every 48 hours.

Effect of different carbon sources

The effect of different carbon sources used in the study was Glucose, dextrose, fructose, and maltose, each and the concentration of 20 g/l, were used in the experiment. the different treatments were performed and inoculated plates were incubated at 20±2°C for 10 days. each treatment was performed in triplicates and radial growth was observed every 48 hours.

Growth media optimization strategies for higher yield of *C. militaris* fruiting bodies

Effect of different grain substrate for fruiting body development

A growth study has been done with different grains such as Brown Rice, Wheat, Millet, Chickpea, and Maize, which were utilized as a carbon source. Grains were washed three times with running tap water and then rinsed with distilled water after that soaked for six hours to remove chaff, dust, and other

particles and autoclave at 121°C for 20 minutes. PD broth 10gm/l, Dextrose 30gm/l, Maltose 10gm/l, KH_2PO_4 1gm/l, $MgSO_4.7H_2O$, 1gm g/l thiamine-HCl (B1) 0.50gm/l, for 1000 ml. The glass bottle was filled with 30 grams of grain and 40ml of liquid media. These bottles were autoclaved for 20 minutes at 121°C. After allowing the glass bottle to cool, *Cordyceps militaris* liquid spawn was poured into each bottle under controlled conditions and then transferred into the growth room, with 75% relative humidity and 20°C temperature. The mycelium's growth was measured regularly. all experiments were done in triplicates (Chun-Li Wang *et al.,* 2017).

Effect of different liquid mediums for fruiting body development

A growth study has been done by using five different liquid mediums with brown rice as grain substrate *viz.* **M1** (glucose 30gm, yeast extract 8gm, peptone 5gm, KH_2PO_4 1gm, $MgSo_4$ 0.5gm, vitamin B1,0.5gm), **M2** (glucose 40 gm, peptone 3gm, KH_2PO_4 2gm, $MgSo_4$ 1gm, ammonium citrate1gm, vitamin B1 0.5gm), **M3** (glucose 40 gm, yeast extract 10gm, peptone 3gm, KH_2PO_4 2gm, $MgSo_4$ 1gm, ammonium citrate1gm, vitamin B1 0.5gm), **M4** (sucrose 40gm, beef extract 3gm, KH_2PO_4 2gm, MgSO4 1gm, ammonium citrate 1gm, vitamin B1 0.5gm), **M5** (glucose 40gm, urea 1gm, yeast extract 10gm, peptone 3gm, KH_2PO_4 2gm, $MgSo_4$ 1gm, ammonium citrate1gm, vitamin B1 0.5gm) The glass bottle was filled with 30 grams of grain and 40ml of liquid media. These bottles were autoclaved for 20 minutes at 121°C. After allowing the glass bottle to cool, *C. militaris.* liquid spawn was poured into each bottle under controlled conditions and then transferred into the growth room, with 75% relative humidity and 20°C temperature. The mycelium's growth was measured regularly. all experiments were done in triplicates.

The mean and standard deviation methods are used to show the differences between the means of individual data. One-way ANOVA with a multiple-range test was used to evaluate differences between the means.

Results and Discussion

The survey, collection, and preservation of culture

Collected fresh fruiting bodies of *Cordyceps militaris* from high altitude sites such as Panchachuli (30° 12' 30.60" N and 80° 25' 23.39" E) and Kalamuni mountain (30.0377° N, 80.1998° E) in the Munsiyari, District Pithoragarh, Uttarakhand, India. The fresh fruiting body was gathered from different sites of Munsiyari, located at a high altitude of approximately 2500m.

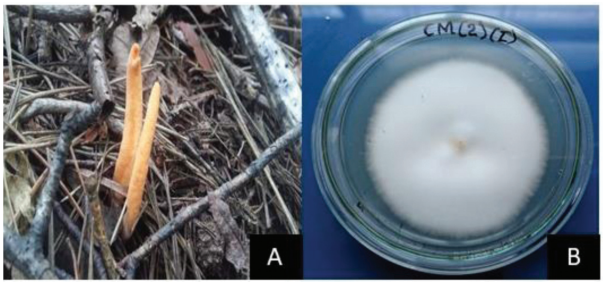

Fig. 1. A. Fresh fruiting body of *Cordyceps militaris*. B-Vegetative growth on CDA media.

Effect of different nutrient media

The radial mycelium growth of *C. militaris* was studied in 10 different media. However, the maximum and minimum growth of fungal culture on different media at the 16th day shown in (Figure-3**)** were CDA 38.99 mm, and CAN 17.60 mm, respectively. Maximum mycelium growth was observed in the CDA medium. Previous studies reported similar finding the maximum mycelium growth was found in OA and MPDA, while SDAY, YMA, and MCM produced sufficient mycelial density (Jae-Mo Sung *et al.,* 2010).

Effect of different temperatures

Mycelium growth of *C. militaris* was observed at various temperatures viz. 5 to 30 in CDA nutrient medium shown in (Figure 4) were used to test, and it was observed maximum mycelium growth of 17.38 mm at 15°C in 10 days. the minimum growth was observed at a temperature of 5°c. Below 15°C, growth was decreased, whereas, above 20°C, it increased. In CDA medium with the temperature of 15°C to 20°C was an appropriate temperature for the mycelium growth of C. *militaris*, Similarly, (Sung *et al.,* 2002) showed that the optimum temperature for mycelial growth of *C. militaris* strains was 25°C. (Dong and Yao, 2005).

Effect of different pH

Mycelium growth of *C. militaris* was observed at various levels pH 4-10 with CDA medium were used to test, and it was observed that the maximum growth of 37.93 mm. At 6pH and pH 4 and pH 10,

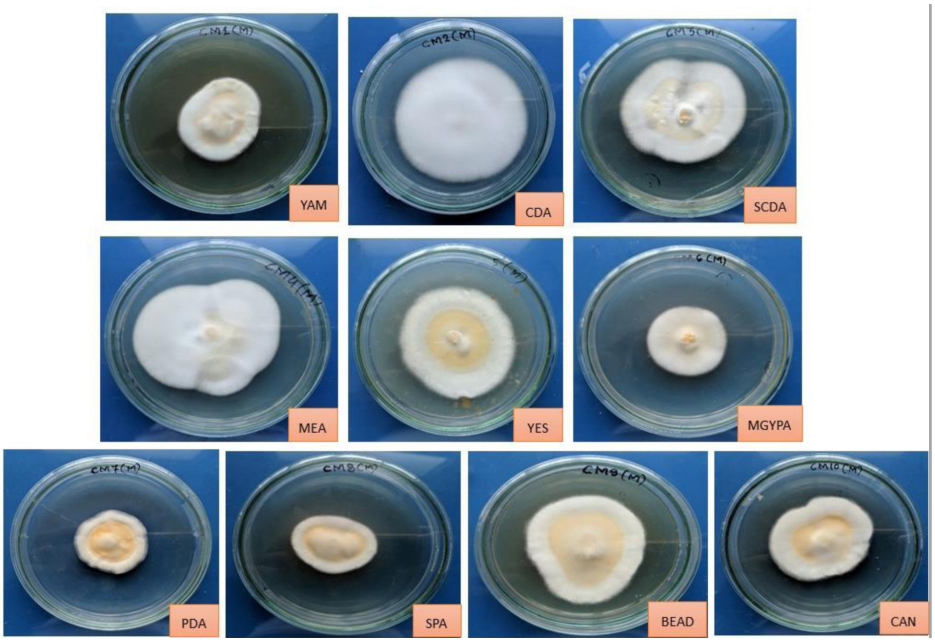

Fig. 2. Radial mycelium growth Study of *Cordyceps militaris* in 10 different nutrient media.

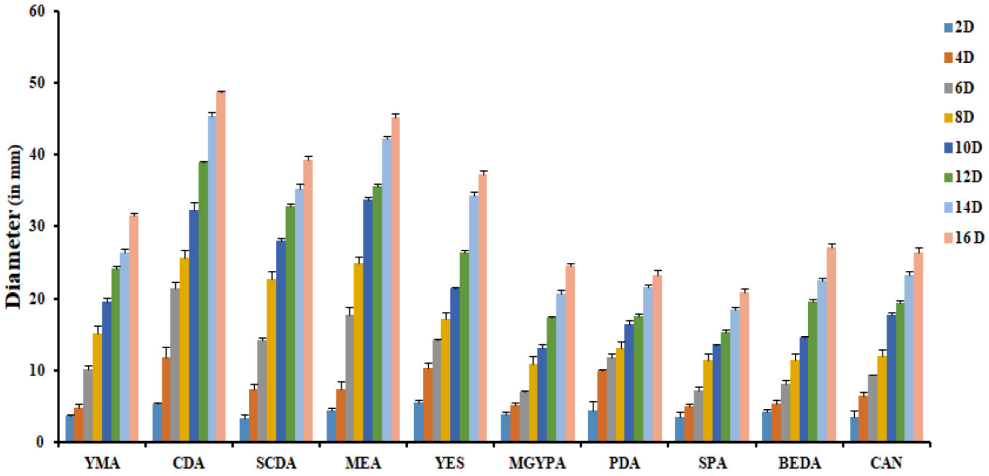

Fig. 3. Graphical representation of mycelium growth of *Cordyceps militaris* on 10 different Nutrient media.

the minimum growth was found. Below pH 5, growth decreased, whereas, above pH 6, it increased as shown in (Figure 5). similarly, the pH value for *C. militaris* mycelial growth was in the range. With different pH levels according to (Sung *et al.*, 2002) and (Park *et al.*, 2001) showed that *C. militaris* mycelia growth was optimum at pH 6, whereas the mycelium developed slowly in according to (Yin and Qin, 2009).

Effect of different carbon sources

Carbohydrate is a key structural component that is also kept in the cell. During the study chosen, 4 different Carbon sources such as glucose, dextrose, fructose, and maltose (20 g/l concentration) were added in the medium for the study of mycelium growth in 10 days at 20°C. The results in (Figure-6) indicated that the mycelium development of *Cordyceps militaris* and significantly found in Glucose and dextrose. According to (Shih *et al.*, 2006) various carbon source, it helps to improve the growth of mycelium.

Effect of different grain substrate for fruiting body development

During this study, were used different grains substrate for the analysis of fruiting body formation of

Cordyceps militaris including brown rice, wheat, millet, chickpea, and maize, used to evaluate their impacts on fruiting body production of *C. militaris*. The results revealed that maximal primordia development of *C. militaris* were found in Brown rice fresh fruiting obtained 47gm and dry fruiting 4.70gm was determined to be the most suitable grain substrate for the fruiting body growth of *C. militaris* and chickpea and millet is much minimum growth in as substrate in compare to fresh fruiting body weight

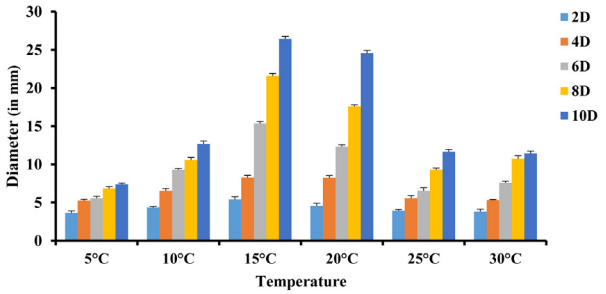

Fig. 4. Graphical representation of mycelium growth of *Cordyceps militaris* on CDA media at different temperatures.

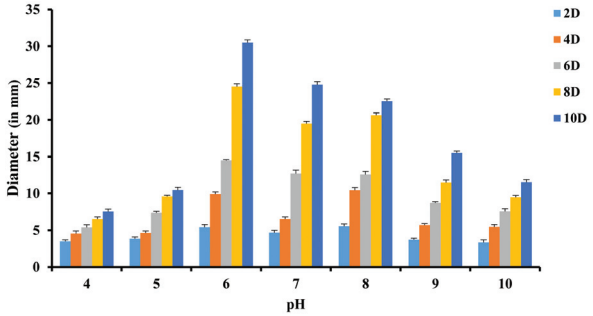

Fig. 5. Graphical representation of mycelium growth of *Cordyceps militaris* on CDA media in different pH levels in 2 days' interval.

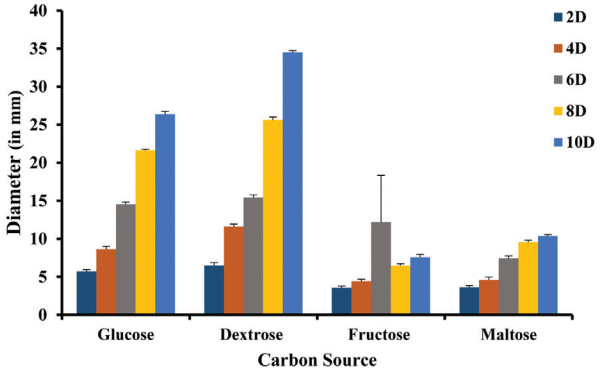

Fig. 6. Graphical representation of mycelium growth of *Cordyceps militaris* on CDA media at different Carbon sources.

and dry weight of fruiting body also found in brown rice.

Effect of different liquid mediums for fruiting body development

the study has been done with 5 different liquid me-

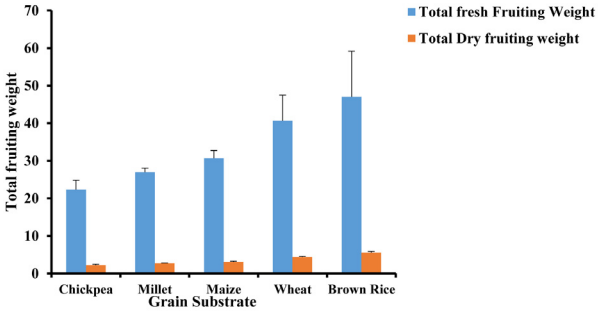

Fig. 7. Graphical representations on different grain substrate for fruiting body development of *Cordyceps militaris*.

Fig. 8. Effect of different grain substrate for fruiting body development of *Cordyceps militaris* A-Brown Rice, B-Wheat Grain, C-Millet Grain, D-Chickpea Grain, E-Maize Grain

diums with brown rice as substrate *viz*. M1, M2, M3, M4, and M5 for the comparative growth analysis of *C. militaris w*ere examined in different liquid media and the results revealed that maximal primordia and the fruiting body were determined is shown in (Figure 9) the fresh fruiting body obtained 52gm and dry fruiting weight is 5.20gm from the M3 media with a combination of Brown rice is the most suitable liquid medium and grain substrate for the high

yield production of *C. militaris* and M1 and M2 with brown rice is a minimum growth was observed.

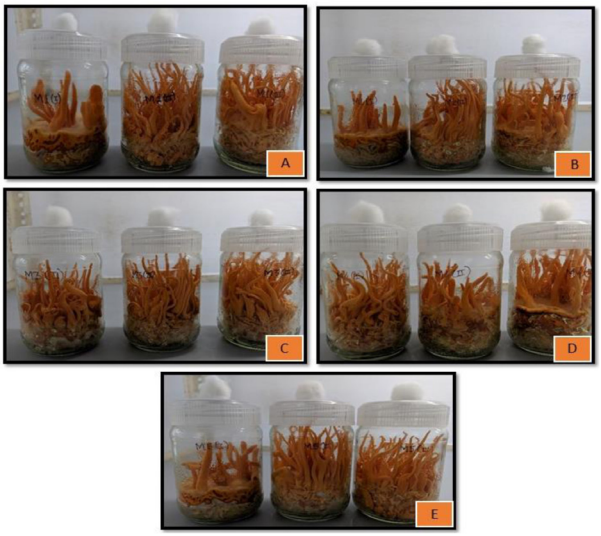

Fig. 9. Graphical representation on different liquid mediums for fruiting body development of *Cordyceps militaris*

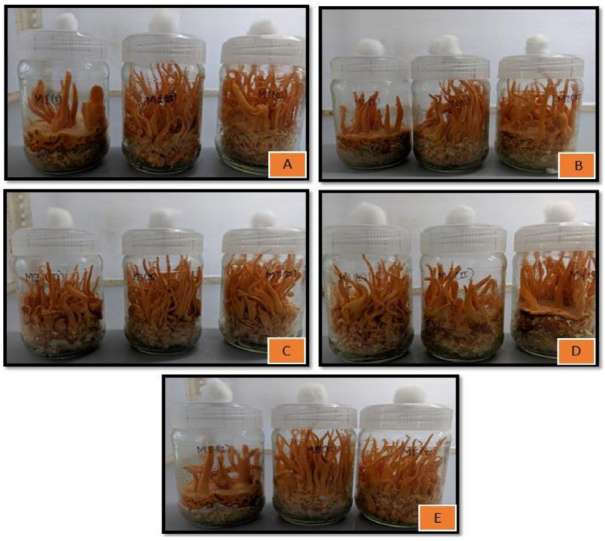

Fig. 10. Effect of different liquid mediums for fruiting body development A-M1 Nutrient media, B-M2 Nutrient media, C-M3 Nutrient media, D-M4 Nutrient media, E-M5 Nutrient media.

Discussion

The most essential component is required for the growth of mushroom production which provides the required nutrients for mushroom mycelia to develop in that study revealed the CDA media was shown to be the most appropriate media for mycelial development of *Cordyceps militaris*. Similarly, several previous research found that PDA was the best medium for the growth of *Macrolepiota procera* and *C. militaris* mycelial development, whereas yeast malt extract medium was best for *M. procera* and *Ganoderma lucidum* mycelial growth. The findings of previous research, as well as the findings of the current study, show that various media increase the mycelium growth and in the study of temperature minimum growth was observed at 5 °C and maximum growth was observed at 15-20°. A solid medium with a temperature of 15 °C to 20 °C was an appropriate temperature for the mycelium growth of *C. militaris*, according to v (Dong and Yao, 2005) many research revealed the *C. militaris* has shown maximum mycelium growth in 20°C to 25°C. *C. militaris* has cultivated at 6.0 and 7.0 pH, respectively, according to (Yin and Qin, 2009). The results indicated that the mycelium development of *C. militaris* was cultured on various carbon sources. Glucose and dextrose were the best carbon sources for mycelium development of *C. militaris* mycelium growth in 10 days at 20 °C. The maximal primordia development of *C. militaris* was found in brown rice was determined to be the most suitable grain substrate for the fruiting body growth of *C. militaris* for the comparative growth analysis of *C. militaris w*ere examined in different liquid media and the results revealed that maximal primordia and the fruiting body were determined in, M3 media with Brown rice is the most suitable liquid medium for the maximum fruiting body growth of *C. militaris*.

Conclusion

To improve the production of valuable components like mycelium and *Cordyceps* fruiting bodies and the culture environment, several experiments were carried out. The most important task is to determine the best cultural conditions and environment for the fungus's growth features. Nutrients media, temperature, pH, carbon source, substrate grain, and liquid media are all significant aspects in the cultivation of *Cordyceps*. the medium (CDA, MEA, and SCDA), The pH ranges from 5 to 7, temperature ranges from 15 to 20 °C, dextrose and glucose carbon source, brown rice with liquid media M3 and culture time all seem to be very suitable for high yield production of *Cordyceps militaris.*

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