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Optimization of nutritional requirements for maximizing the mycelial growth and biomass production of *Ophiocordyceps sinensis*

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ABSTRACT

Ophiocordyceps sinensis* (ester while *Cordyceps sinensis*) an entomo-pathogenic fungus also known as Chinese caterpillar fungus is traditionally valued in Chinese medicine as it secretes lots of metabolites of pharmaceutical importance. However, this mushroom required diverse nutritional requirements for fast growth and maximum biomass production and fruiting body development. In this regard, a study was initiated to test different carbon, nitrogen, minerals, vitamins and chitin sources to enhance the growth and biomass production of *O. sinensis* under liquid fermentation. Among the carbon and nitrogen sources tested, sucrose (3%) and beef extract (0.5 %) showed maximum mycelial growth and biomass production (90.00 mm, 8.13g, and 8.17 g dry weight /L in 18 d). Also, the best mineral sources are K_2HPO_4 and zinc chloride (0.2 %) that enhanced the maximum mycelial growth and biomass production (90.00mm, 8.20, and 8.12 g/L on 18 d respectively). Following this, the suitable vitamin (0.01 %) and chitin sources (0.1 %) like folic acid and dried powder of rhinoceros grub has enhanced the growth and biomass production of *O. sinensis* (90.00mm, 8.31, and 8.69 g/L on 18 d, respectively). This study paves way to enhance the metabolites secretion, biomass production, and fruiting induction of *O. sinensis.

Keywords: *O. sinensis*, mycelium growth, biomass production, metabolite production

Ophiocordyceps sinensis, an entomo-pathogenic fungus also known as Chinese caterpillar fungus is traditionally valued in Chinese medicine. (Buenz *et al.,* 2005). In nature, the fungus parasitizes on larvae of ghost moths (*Hepialus armoricanus*) and converts each larva into sclerotium, where the fruiting body emerges (Huang and Ohga, 2018). It is one of the most valuable medicinal fungus known by pharmaceutical industries possessing potent biologically active substances. *O. sinensis* is known to produce diverse biologically active compounds such as adenosine, cordycepin, cordymin, D-mannitol, and exopolysaccharides is having antitumor, anti-inflammatory, antioxidant, anti-diabetes, antifatigue, and anticancer activity (Das *et al*., 2010; Zhang *et al.,* 2010 and Cui *et al.,* 2018) used in pharmaceutical industries. In addition, these compounds have antifungal and antinemic activity (Sangeetha *et al.,* 2015a, b, and Akshaya *et al.,* 2021). However, the cultivation and production of the fruiting body is a great challenge due to diverse nutritional requirements. Also, the fruiting body production is dependent on mycelial vigour which in turn is dependent on the nutrient composition of culture media. The production of mycelium in submerged conditions plays a crucial role in the fermentation process and extraction of useful biomolecules (Ren and Yao, 2013). Furthermore, it is well known that

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carbon and nitrogen sources are the major key component for mycelial growth, biosynthesis of enzymes for primary and secondary metabolism, and synthesis of biological compounds in submerged liquid fermentation (Wen *et al*., 2013). In addition, minerals and vitamins sources are required for the growth of mycelium and help in proper hyphal development in *Cordyceps sinensis* (Kang *et al*., 2014). Hence, the present study was attempted to investigate the efficacy of different carbon, nitrogen, minerals, vitamins, and chitins sources in enhancing the mycelial growth to harvest maximum biomass of *O. sinensis* in order to tap metabolites.

MATERIALS AND METHODS

Standardization of cultural conditions of *O. sinensis*

The culture of *Ophiocordyceps sinensis* (isolate no.1220) obtained from the Forest Research Institute (FRI), Dehradun, India was used for this study.

Growth characteristics and biomass production on solid and liquid media

The mycelial growth of *O. sinensis* was evaluated in five different growth media *viz.,* Mushroom Complete Medium (MCM); Potato Dextrose Agar (PDA); Sabouraud Dextrose yeast Agar (SDAY), Asthana & Hawkers medium (AHM), and Malt Extract Agar (MEA). The inoculated Petri dishes were incubated at 18°C. The radial growth of the mycelium was measured at 3 days intervals till full growth was attained in a Petri dish (90 mm). Five different broths mentioned above were prepared and the pH was adjusted to 5.5. The sterile broth @ 100 ml in 250 ml conical flask was inoculated with 9 mm diameter of mycelial discs of *O. sinensis.* The flasks were placed in an incubator cum shaker (120 rpm) maintained at 18°C for different days *viz*., 5,10,15, and 20d. After completion of different periods of incubation, the mycelial mat was removed from the broth and washed repeatedly with sterile distilled water to eliminate the chemical residues, dried at 60°C, and weighed separately.

Effect of carbon, nitrogen, and mineral sources of nutritional mycelial growth

Based on the above results, SDAY broth was selected for further studies owing to better mycelia growth of *O. sinesis.* The carbon, nitrogen, mineral, and vitamin contents in the basal SDAY medium (Dextrose) 40g, Peptone 10 g, Yeast extract 10 g, Agar 20 g, and Water 1000 ml) were replaced with different sources to find out the most efficient source to enhance the mycelial growth and biomass production of *O. sinensis*.

Carbon sources

Carbon sources of nutrients *viz.,* mannitol, maltose, sucrose, sorbitol, and fructose were substituted separately to the basal SDAY (liquid) at the rate of 3 per cent (w/v) to replace Dextrose. The 10 days old mycelia discs (9 mm diameter) of *O. sinesis* were cut with a sterilized cork borer and aseptically inoculated on 250 mL conical flasks (100 mL broth) of SDAY broth containing different carbon sources and incubated at 25°C. The basal SDAY broth served as control.

Nitrogen Sources

Five different organic and inorganic nitrogen sources *viz*., Ammonium nitrate, Ammonium sulphate, Potassium nitrate, Sodium nitrate, and Beef extract were added separately to SDAY (liquid broth) at the rate of 0.5 per cent (w/v) to replace the nitrogen source. The 9 mm diameter culture discs (10 d old) of *O. sinesis* were cut with a sterilized cork borer and aseptically inoculated on 250 mL conical flasks (100 mL broth) of SDAY broth containing different nitrogen sources and incubated at 25°C. The basal SDAY broth served as control.

Mineral supplements (macro and micro)

Five different macro mineral sources *viz*., $CaCl_2$, $2H_2O$, KH_2PO_4, K_2HPO_4, $MgSO_4$, $7H_2O$ and NaCl and five micro mineral sources *viz.,* $CuSO_4$, $5H_2O$, $FeSO_4$, $7H_2O$, $MnCl_2$, $4H_2O$, $MnSO_4$ and $ZnCl_2$, were used at 0.2 per cent to the basal SDAY broth. The culture discs of 9 mm diameter (10 d old) were cut with a sterilized cork borer and aseptically inoculated on 250 mL conical flask (100 mL broth) containing mineral replaced SDAYand incubated at 25°C. The basal SDAY served as control.

Vitamin Sources

Five different vitamin sources *viz*., Folic acid, D-biotin, Thiamine, Nicotinic acid, Riboflavin were used at 0.01 per cent to the basal SDAY broth. The 9 mm culture discs of *O. sinensis* (10 d old) were cut with a sterilized cork borer and aseptically inoculated on 250 mL conical flask (100 mL broth) basal SDAYand incubated at 25°C. The basal SDAY served as control.

Effect of different chitin source

Five different chitin sources *viz.,* chitin, colloidal chitin, grab shell chitin, the grub of rhinoceros beetle, and cricket insect was used at 0.1 per cent. The 9 mm culture discs of *O. sinensis* (10 d old) were cut with a sterilized cork borer and aseptically inoculated on 250 mL conical flask (100 mL broth) containing basal SDAY and incubated at 25°C. The basal SDAY served as control.

Statistical Analysis

The design of experiments i.e. CRBD and statistical analyses were followed as suggested by Gomez and Gomez (1984). Statistical software AGRES (Developed by the Department of Physical science, TNAU, Coimbatore) was used for the analyses of the data.

RESULTS AND DISCUSSION

Growth and biomass production of *O. sinensis* in different media

Among the five different growth media tested for mycelial growth, SDAY supported the maximum growth of the *O. sinensis* (90.00 mm within 15d), followed by MCM (83.00 mm in 15d) and PDA (65.00 mm in 15 d) were appended in Table 1. In PDA medium, initially, the mycelial growth was whitish in colour, turned into dull black. In SDAY medium, initially creamy white mycelium observed, finally turned white. In the MCM medium, the colony colour initially showed light yellow which turned changed to dark. In the MEA medium, the colony colour initially changed from white to dark. In Asthana & Hawker's medium (AHM), the mycelial colour though initially appeared light whitish colour colony appeared finally changed into dull white (Fig 1). Growth media is known to influence the cultural characters of several fungi (Kim and Yun, 2005). Standardization of nutritional requirements and media supplements support the growth stage and influence the production of several bioactive compounds, as well as useful secondary metabolites (Shih *et al.,* 2007; Das *et al.,* 2010a, and Lim *et al.,* 2012). In the case of *O. sinensis,* several media compositions have been proposed to influence the mycelial growth in submerged cultures (Xu *et al.,* 2002; Dong and Yao, 2005; Kim and Yun, 2005). In the present study, the maximum radial growth of the colony and biomass production of *O. sinensis* was supported by SDAY followed by MCM and PDA medium and these findings were in line with Arora *et al.* (2013). The colour change is an indicator of the nutritional status of the growth medium, sometimes due to the reduction of specific nutrients which indicates active utilization and the stage of growth of the fungus (Sutthisa and Sanoamuang, 2014).

The biomass production of *O. sinenis* in five different liquid broths were observed at different days

Fig. 1. Growth of *Ophiocordyceps sinensis* on different media

Table 1. Radial mycelial growth of *O. sinensis* on different growth media

Media	Radial mycelial growth (mm)					
	3 d	**5 d**	**7 d**	**9 d**	**12 d**	**15 d**
Potato dextrose agar	7.4[b]	14.20[c]	34.20[b]	41.20[c]	53.00[c]	65.00[c]
Mushroom complete media	7.0[c]	18.10[b]	32.20[c]	42.10[b]	65.00[b]	83.00[b]
Sabouraud's dextrose yeast agar	8.0[a]	22.00[a]	39.60[a]	50.50[a]	70.00[a]	90.00[a]
Asthana & Hawker's medium	4.5[e]	11.20[e]	28.20[e]	36.10[e]	40.00[e]	52.00[e]
Malt yeast extract	6.9[d]	12.20[d]	30.10[d]	39.20[d]	50.00[d]	62.00[d]
CD (p = 0.05)	0.10	0.33	1.61	1.92	2.20	3.81

Values are the mean of three replications.
Means followed by a common letter are not significantly different by one-way ANOVA.

interval. The results revealed that after the 20th day of incubation, the maximum biomass yield was obtained in SDAY broth (12.10 g/L) followed by MCM broth (10.30 g /L) and PDA broth (8.10 g/L). However, malt yeast extract and Asthana & Hawker's broth also supported fairly good biomass production (7.00 g/L and 6.00 g/L, respectively) (Table 2). The colony colour of *O. sinensis* in SDAY broth was initially creamy white, turned into white on 12 d after inoculation which shows that active metabolism

has taken place. Similar results were noticed during liquid-state fermentation also over a period of 18 d of incubation. The colour change of the growth medium into dark could be correlated with the production of phenolics, terpenoids, and several oxidative enzymes having different kinds of biological activity. This kind of variation in colony morphology and pigmentation of growth media during the growth of *O. sinensis* were reported earlier by Garbyal *et al.* (2004), Shi *et al.* (2009), Xie *et al*. (2010) and Marchbank *et al*. (2011).

Table 2. Biomass yield of *O. sinensis* in different growth media

Broth	Mycelia dry weight (g/L)			
	5 d	10 d	15 d	20 d
Potato dextrose broth	1.00c	3.90c	7.50c	8.10c
Mushroom complete broth	1.40b	4.50b	8.50b	10.30b
Sabouraud's dextrose yeast broth	1.80[a]	5.00b	10.10c	12.10[a]
Asthana & Hawker's Broth	0.10e	2.00e	5.00e	6.00e
Malt yeast extract Broth	0.70d	3.30d	6.80d	7.00d
CD (p = 0.05)	0.0249	0.0849	0.3099	0.2796

Values are the mean of three replications.
Means followed by a common letter are not significantly different by one-way ANOVA

Standardization of nutritional requirements for maximum biomass of *O. sinensis*

Effect of carbon source

To find out the best carbon sources for the induction of mycelial growth and biomass production, *O. sinensis* was cultivated for 18 d in SDAYsupplemented with various carbon sources. The results showed that when sucrose was used as the carbon source, both mycelial growth and biomass production was found to be maximum (90.00 mm and 8.13g dry weight /L in 18 d) followed by mannitol (70.00 mm and 6.10 g dry weight /L in 18 d) and sorbitol (63.60 mm and 5.33g dry weight /L in 18 d). Both fructose and maltose supported reduced mycelia growth and biomass production (53.60 mm, 4.63 g dry weight /L and 43.00 mm, 4.40 g dry weight /L, respectively in 18 d) (Fig 2a.). All fungi require carbon-containing substances such as sugars as sources of energy for initial growth. Besides, sucrose, is a major sugar component of photosynthetic plants and also composed of two molecules α-D-glucopyranozyl and α-D-fructofuranozit which might enhance the growth of *C. sinensis* (Dong and Yao, 2005). The present study revealed that the highest mycelial yield and biomass were obtained in sucrose followed by mannitol. Similar results were obtained by Nam *et al.* (2019) where they reported that glucose and sucrose increased the mycelial growth of *Cordyceps militaris*.

Effect of nitrogen source

The basal SDAY broth was replaced with different nitrogen source supplementation, at the rate of 0.5 per cent (w/v). The results of the experiment revealed that the maximum mycelial growth and biomass production was supported by beef extract as the sole nitrogen source when compared to others (Fig 2b). In this treatment, an average of 90.00 mm linear growth and 8.17 g dry weight /L were recorded 18 days after incubation. The second-best nitrogen source was potassium nitrate as it supported 67.20 mm growth and 7.72 g of mycelial dry weight /L followed by ammonium nitrate and ammonium sulphate (61.20mm growth, 59.40 mm growth, and 6.60 and 5.69 g of mycelia dry weight /L). The inorganic nitrogen source *viz*., sodium nitrate poorly supported the mycelial growth and biomass production (47.40 mm and 4.83g biomass / L) on a dry weight basis. Nitrogen-containing substances are required for the formation of amino acids to build proteins and other essential components like nucleotides (Purines). During the current investigation it was observed that among all the organic and inorganic nitrogen sources, *O. sinensis* had a preference for beef extract as observed by maximum mycelial growth and biomass production followed by potassium nitrate. Organic nitrogen sources were significantly superior and more productive than inorganic nitrogen sources. In support

of the present findings, Tuli *et al.* (2014) had indicated that yeast extract was very effective for increased mycelial growth and cordycepin production (709 mg /L). Thus, organic sources of nitrogen supported luxuriant mycelial growth and biomass compared to the inorganic source.

Effect of mineral (macro and micro) sources

The influence of various mineral sources of nutrition on mycelial growth and biomass production of *O. sinensis* revealed that among the various mineral sources, K_2HPO_4 supported maximum mycelial growth and biomass (90.00mm and 8.20 g/L on 18 d) followed by MgSO4, $7H_2O$ (78.00 mm and 7.60 g/L), KH_2PO_4 (72.50 mm and 7.25 g/L), and $CaCl_2$, $2H_2O$ (69.40 mm and 6.45 g/L) whereas NaCl supported least mycelia growth (48.71mm and 6.25 g/ L) (Fig 2c). In the case of microelements, zinc chloride recorded maximum mycelial growth and biomass (90.00 mm and 8.12g/L on 18th d) followed by $CuSO_4$, $5H_2O$ (76.50 mm and 7.31), $FeSO_4$, $7H_2O$ and $MnCl_2$, $4H_2O$ (65.00 mm, 60.00 mm and 7.15g/ L, 6.25 g/L). The lowest mycelial growth and biomass production was observed in manganese sulphate (58.00 mm and 6.20 g /L) supplemented medium (Fig 2d.). Besides, furnishing utilizable macro and minor minerals, phosphate and potassium ions salts might also act as useful buffers and exert a controlling influence over the pH changes in the growth medium regarding influencing primary and secondary metabolic pathways in the fungus (Kaur, 2011). Additionally, minerals sources like $MgSO_4$, $7H_2O$ and K_2HPO_4 enhanced the production of the fruiting body and increased the level of cordycepin secretion in *C. militaris* (Wen *et al.,* 2013). These findings were similar to our study were K_2HPO_4, and $CuSO_4$ at 0.1 per cent had a significant impact on mycelial growth and biomass production.

Effect of vitamin sources

The influence of various vitamin sources on the mycelial growth and biomass production of *O. sinenis* revealed that folic acid exhibited maximum mycelial growth and biomass (90.00 mm growth and 8.31 g/L on 18th d), followed by thiamine and D-biotin (73.55 mm,70.00 mm, and 7.05g/L, 6.41 g/L), and nicotinic acid (56.20 mm and 5.90 g/L). The lowest mycelial growth and biomass production was observed in riboflavin (51.20 mm growth and 5.13 g dry weight/ L) supplemented broth (Fig 2e). Additionally, vitamin sources also enhanced mycelial growth and biomass production at very low concentrations also have a catalytic function in the cell constituents of coenzymes. In the present study, the folic acid infusion to the growth medium recorded the highest biomass production and mycelial growth of *O. sinensis*. These findings are in agreement with the findings of Singh *et al.* (2012) who reported that Folic acid and D-Biotin enhanced the biomass production of *O. sinensis*. However, Kang *et al.* (2014) reported that the maximum cordycepin production in *C. militaris* was the basal medium supplemented with vitamin B1 (VB1). Contrary to our study, Dang *et al.* (2018) reported that thiamine HCl (B1) and nicotinic acid induced the mycelial growth and biomass production of *C. militaris*. The present investigations conclude that vitamin sources will have a strong effect on mycelial growth, fruiting bodies production of *O. sinensis.*

Effect of chitin sources

The dried chitin powder from grub of rhinoceros beetle (*Oryctus rhinoceros*) used as the chitin source supported maximum mycelial growth and biomass production (90.00 mm and 8.69 g/L on 18d) followed by powdered supplement of mole cricket (81.00 mm and 8.28 g/L on 18 d) and crab shell chitin (79.00 mm and 7.56 dry weight /L in 18 d). The minimum growth and less biomass production were observed in colloidal chitin (Sigma Aldrich, USA) and chitin (Nitte Gelatin, Cochin) supplements (65.00 mm, 60.00 mm and 6.87g/ L, 6.46g/L on 18, respectively) (Fig 2f.). It is known that chitin also the second most polysaccharide after cellulose, is present in cell walls of several fungi, exoskeletons of insects, and crustacean shells. In the

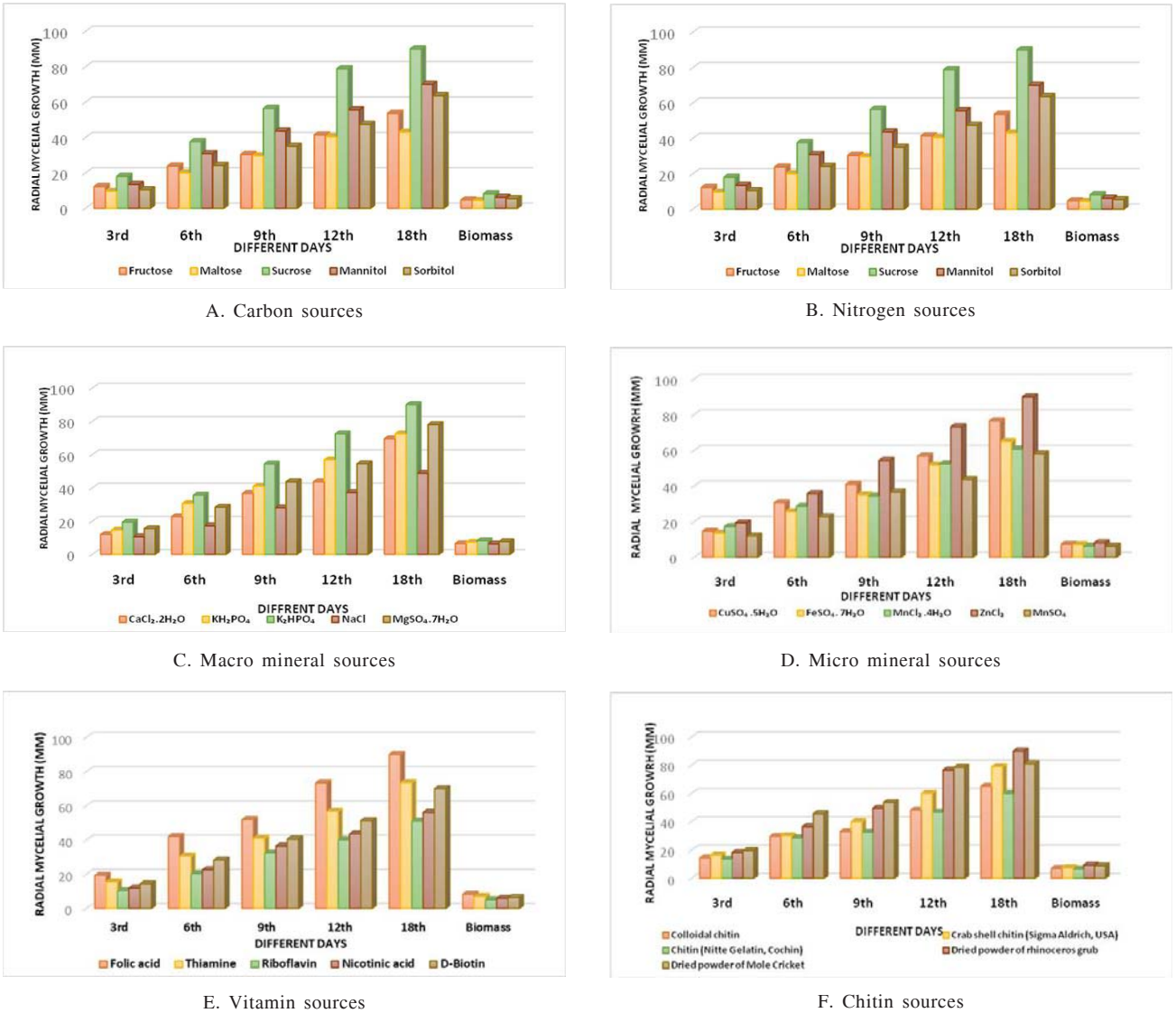

Fig. 2. Nutrient requirements of biomass production

existing study different chitin sources when added in basal SDAY broth enhanced the mycelial growth and biomass production of *O. sinensis*. The dried powder from the grub of the rhinoceros beetles enhanced the mycelial biomass production of *O. sinensis* because the exoskeleton of the grub cuticle is made up of chitin. Wang *et al*. (2021) reported that the hypoxia (*Vitreoscilla* hemoglobin) plays an important role in the growth of the mycelial, chitinases and protease production, biomass production, and the synthesis of bioactive compounds in *C. militaris*.

Hence, from the present study, it is concluded that the selection of an appropriate nutrient medium and its composition play a crucial role in the biomass growth of *O. sinensis* and to induce the extracellular secretion of bioactive compounds or secondary metabolites.

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