International Journal of Science and Research (IJSR)
ISSN (Online): 2319-7064
Impact Factor (2012): 3.358

Optimization of Nutritional Necessities for *in vitro* Culture of *Ophiocordyceps Sinensis*

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Abstract: ***Ophiocordyceps sinensis has long been a core of traditional medicine which enhances the stamina and potency. Due to its less availability in nature and increasing demand, it needs attention for its in vitro culture to fulfill the future demand. This study revealed that in vitro culture using synthetic media along with various nutritional supplements gives promising mycelial yield. In the present work various nutritional supplements, including four carbohydrates sources, eight nitrogen sources, six vitamins, five macro minerals and four micro minerals were studied for in vitro mycelial culture of O.sinensis. This study also revealed the optimized quantity of nutitional suppliments for high yield. The study of this work revealed that mycelium yield was significantly higher on sucrose among the carbon sources. Beef extract and yeast extract showed significantly higher mycelial yield among nitrogen sources. Organic nitrogen sources were significantly more productive then inorganic nitrogen sources. Yield obtained with folic acid was significantly higher among vitamin sources used. In all micro nutrients and macro nutrients, calcium chloride and zinc chloride were significantly higher than other variables used. The nutritional requirements for mycelial growth of Ophiocordyceps sinensis in synthetic liquid media (PD broth) were investigated. The concentration of sucrose, beef extract, folic acid, calcium chloride and zinc chloride were optimized. The effects of this optimized medium composition were studied. Mycelial dry weight 12.08 g/L was obtained with this optimized medium which is significantly higher than PD broth (3.85g/L) medium. These results provide a basis for further physiological study and industrial fermentation of the fungus for their better future use in nutraceutical industry and medicinal field.***

Keywords: *Ophiocordyceps sinensis,* Synthetic media, Mycelial yield, Nutritional necessities, Agitation.

1. Introduction

Ophiocordyceps sinensis **[1]** (syn. *Cordyceps sinensis (Berk) Sacc.*) an Ascomycetes fungus, is a parasite on larvae of *Thitarodes* (*Hepialus*) moths **[2]**. It is one of the most valued traditional Chinese medicines. It is popularly referred to as the Chinese caterpillar fungus or 'Dong Chong Xia Cao' (summer grass winter worm) in Chinese, or 'Hia Taso Tong Tchong'and Hea Tsaon Tsong Chung in early English Translations **[3].** *O. sinensis* is parasitic on larvae of insect *Thitarodes sp.* The scientific name for the sexual stage including stalked fruiting-body is termed as *O. sinensis* whereas its asexual mycelium culture is known as *Hirsutella sinensis* **[4].** In late summer the mycelia invade and replace the internal organs of host larvae with thickened fungal tissue known as endosclerotium **[5].** When the environmental temperature rises fruiting-body (stroma) sprouts from the dorsal surface of prothorax of infected larvae, develops gradually and matures consequently in late summer (June-August) **[6].** The *Ophiocordyceps sinensis*, caterpillar-shaped Chinese medicinal mushroom **[4;7]** is confined to the high Himalayan Mountains in China, Tibet, Nepal and India, at an altitude ranging from 3000 to 5000 m **[8]** or in Asian high altitude grassland ecosystems **[9]**. The genus *Thitarodes* seems to be restricted to eastern parts of Asia with a geographical range stretching from Nepal in the west, southwards into Myanmar, and towards the east into Taiwan, Japan, and northeastern parts of Russia. The most recent inventory of the Hepialoidea **[10]** lists 51 species belonging to *Thitarodes*, the majority of which have only relatively recently been described from China, particularly Yunnan and Tibet **[11].** There are more than 700 species of this fungi present, of which about 300 species have been reported to produce a fruiting body, namely *Cordyceps* mushroom **[12; 13]**. It is one of highly potential medicinal mushrooms in the world **[14].** It helps in curing various diseases. It acts as anti-bacterial **[13],** immunomodulatory **[15]**, immunosuppressive **[16]**, anti-complementary [**17]**, anti-tumour **[18]**, anti-inflammatory [**19]** anti-oxidant **[20],** anti-diabetes **[22],** anti-fatigue **[23]** anti-aging [**24].** It is known as "panacea of all ill "due to its high efficacy and potency **[25].** It contains many bioactive molecules like polysaccharide **[15; 16]**, Cordycepin **[17],** adenosine [**18]**, cordymin **[20]** and ergosterol **[24]**.

In recent years, DongChongXiaCao has been regarded as the Himalayan Viagra which has caused the price to reach US$ 6.77 per piece of wild medicine **[22]**. As of August 2012, the price per gram of wild DongChongXiaCao in Beijing was reported to be US $111,560 per kg [2]. It is very rare due to its confined geographic distribution and overexploitation in recent decades **[16].** The fungus has been officially classified as an endangered species by the Chinese government since **1999 http://www.gov.cn/gongbao/content/2000/content_60072.htm**).

Natural production of fungus is limited and annual yield has been declining continually over recent a year **[26]** so it stimulated the interest for *in vitro* culture. Fruiting bodies has not been developed successfully so the production of mycelia by *in vitro* culture is only a promising alternative. So, standardization of nutritional supplements with optimum quantities is required for large scale production of *O.sinensis* in less time with minimum uses of resources.This *in vitro* culture could be a good future in the realm of nutraceutical industry as well as in medicinal field.

In the present work nutritional requirements were studied. The effect of carbon, nitrogen, vitamin and mineral sources

on mycelia growth was investigated and an optimized media (with nutrient supplements) were designed for further production of mycelium in large quantities. The result of this study will facilitate research on mass production of such costly fungus *O. sinensis* under defined culture conditions.

2. Material and Method

Natural *O. sinensis* specimens were collected from Laspa region (N-30 17' 06.59^0 and E-80 11' 27.2^0) of district Pithoragarh, Uttarakhand (India). Pure culture was obtained by culturing tissues from stroma region of wild *O. sinensis* **[27].**

The source of chemical used
The chemicals used were media PD broth (HIMEDIA), all carbohydrate Loba Chemicals (Mumbai, India), and all nitrogen sources Loba Chemicals (Mumbai, India), all vitamins Sigma-Aldrich (Steinheim, Germany) and minerals Loba Chemicals (Mumbai, India)

Instrument used
Bioreactor (Bio Flo 115 New Brunswick, Eppendorf Company), Incubators shaker, (Innova 42, New Brunswick scientific, Eppendorf Company) and pH meter (Microproccer pH meter, Naina Solar Ltd.), Lyophilizer (Freeze dryer)

The media preparation and culture conditions
The media is prepared by using Potato dextrose broth (24g/L) and the medium (200 ml) was added to a 1000 ml Erlenmeyer flask and autoclaved at 121 ºC for 20 min. and pH 5.5 maintained with 1N NaOH and 1N HCL. The medium was inoculated with 10 ml of seed culture mycelium and incubated on incubator shaker at 200 rpm and temperature maintained at 10ºC for 30 days. The mycelium was harvested by centrifugation for 20 min at 6000 *rpm* to separate it from liquid medium. After repeated washing pellets with distilled water and drying was done in Lyophilizer for 48 hour and then dry weight of mycelium was obtained.

2.1 Effect of Carbon Source

All fungi require carbon-containing substances such as sugars as sources of energy. Carbohydrates are generally the preferred carbon source. Fungi can readily absorb and metabolize a variety of soluble carbohydrates, such as glucose, maltose, sucrose, and fructose. Glucose and fructose a simple carbohydrate readily assimilated by the fungus for its nutritional needs. Fungi are also characteristically well equipped to use insoluble carbohydrates such as starches, cellulose, and hemicelluloses, as well as very complex hydrocarbon like lignin. In this experiment two monosaccharide (glucose and fructose) and disaccharide (sucrose and maltose) were tested. Culture medium was prepared by using PD Broth (24g/L) then added each carbon sources 30 g/L (this amount is optimized after several experiments for carbon sources) into the broth medium and all other parameters were maintained as mentioned in media preparation and culture condition. A medium free from carbohydrate source served as a control. Each treatment was replicated three times.

2.2 Effect of Nitrogen Source

Nitrogen-containing substances are required to build proteins and other essential components. Nitrogen is required for the formation of amino acids and nucleotides (Purines). Many fungi utilise nitrate, but a few require ammonium, or even amides, amino acids or peptides. Relatively low nitrogen content will slow the rate of degradation, because of the demand for protein. Conversely, if the organic matter has high protein content, then the protein may be used as a source of N and organic carbon. Once C and N requirements are met, the rate of growth is determined by availability of other minerals. In this experiment six inorganic (Ammonium chloride, Ammonium nitrate, Ammonium sulphate, Potassium nitrate, Sodium nitrate, and urea) and two organic (yeast extract, Beef extract) nitrogen sources were tested. Culture medium was prepared by using PD broth (24g/L) in same manner as mentioned in medium preparation and culture conditions and added 4 g/L of each nitrogen sources in separate flask. A medium lacking any nitrogen source served as a control.

2.2 Effect of Vitamin Source

Vitamin sources are required to build the growth of mycelium. Vitamin helps in proper development of hyphae and also promotes growth of mycelium. In this experiment six vitamin (Folic acid, D-biotin, Thiamine, Nicotinic acid, Riboflavin and Pyridoxine) sources were used. Culture medium was prepared by using PD Broth (24g/L) then added each vitamin sources (10mg/L) to media and autoclaved (only Folic acid and Thiamine is sterilised by using 0.22µm aperture filter prior to added into the medium **[28]** and other parameters were same as medium preparation and culture condition.

2.3 Effect of minerals (macro and micro) sources

In this experiment five macro nutrient sources ($CaCl_2.2H_2O$, KH_2PO_4, K_2HPO_4, $MgSO_4.7H_2O$, and NaCl) and four micro nutrient ($CuSO_4$.$5H_2O$, $FeSO_4$. $7H_2O$, $MnCl_2$.$4H_2O$ and $ZnCl_2$) sources were studied. Culture medium was prepared by using PD Broth (24g/L) then added each macro mineral source 1g/L and micro 500 mg/L to media and all other parameter was maintained as mentioned in media preparation and culture condition .

2.4 Optimized Medium Composition

The optimized medium contains 30 g/L sucrose, 4 g/L beef extract, 10 mg/L folic acid, 1 mg/L calcium chloride, 500 mg/L zinc chloride ,along with 24g/L PD broth. pH at 5.5, temperature 10ºC and agitation at 200 rpm was maintained. Dry weight was obtained same as mentioned in media used and culture condition. This experiment was performed in triplicate.

2.5 Statistical Analysis

The data were analysed by balanced anova by using crop stat software and significant differences were analysed by (Duncan's multiple range test) LSD (at $P<0.05$).

3. Results and Discussion

a. Carbon source

There were significant effects of carbon source on the mycelial growth of *O. sinensis* (Table & Fig1). Among the carbon sources tested, the highest mycelial yield was obtained with Sucrose then maltose follwed by glucose. It appeared that disaccharides had stronger effects on mycelial growth than the monosaccharides tested. Weak growth was observed in the media with fructose with respect to all carbon sources used. From the practical point of view, sucrose is a good candidate for the carbon source because of its ease-of-use and low cost, compared with other carbon sources. Therefore, sucrose was selected as the good carbon source in the following experiments among all sources.

Table1: Effect of carbon sources on mycelium yield of *O. sinensis*

C-sources	Amount used (g/L)	Dry weight (g/L)
Monosaccharide		
Glucose	30	4.7 b
Fructose	30	4.2 c
Disaccharide		
Sucrose	30	5.6 a
Maltose	30	4.8 b
Check(No carbon)	-	3.3 d

(LSD at 5% is 0.3 and SE 0.1)
The mean Values indicated by different letters are significantly different by LSD test ($p<0.05$)

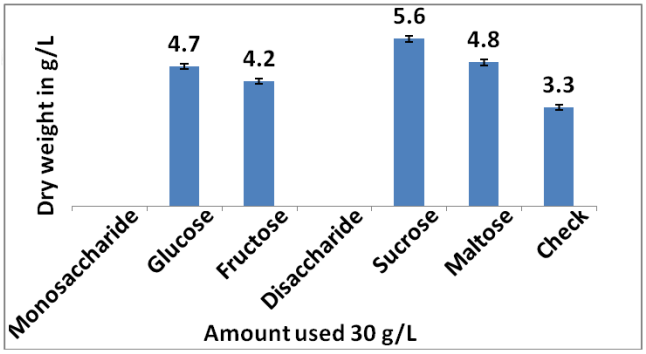

Figure 1: Dry weight obtained by using different carbon sources

b. Nitrogen source

Among 7 nitrogen sources examined, beef extract was the most effective for the mycelial growth of *O. sinensis* (Table 2). The mycelial yield in the media supplied with beef extract was significantly higher than that of any other nitrogen sources. Nitrates (ammonium, potassium and sodium nitrates) were better than ammoniums (ammonium chloride and ammonium sulfate) for the mycelial growth of *O. sinensis*. Urea could not be utilized by the fungus and had a negative effect on its growth because the dry weight of mycelium from the media with urea was even lower than that in the control which did not contain any nitrogen.

Table 2: Effect of Nitrogen sources on mycelium growth of *O. sinensis*

N-sources	*Amount used (g/L)*	*Dry weight (g/L)*
Inorganic		
Ammonium chloride	4	2.4 e
Ammonium nitrate	4	3.4 c, d
Ammonium sulphate	4	3.3 d
Potassium nitrate	4	3.7 c
Sodium nitrate	4	3.5 c, d
Urea	4	2.0 f
Organic		
Yeast Extract	4	5.2b
Beef extract	4	6.5 a
Check (No nitrogen)	-	3.1 d

(LSD at 5% is 0.3 and SE 0.1)

The mean Values indicated by different letters are significantly different by LSD test ($p<0.05$)

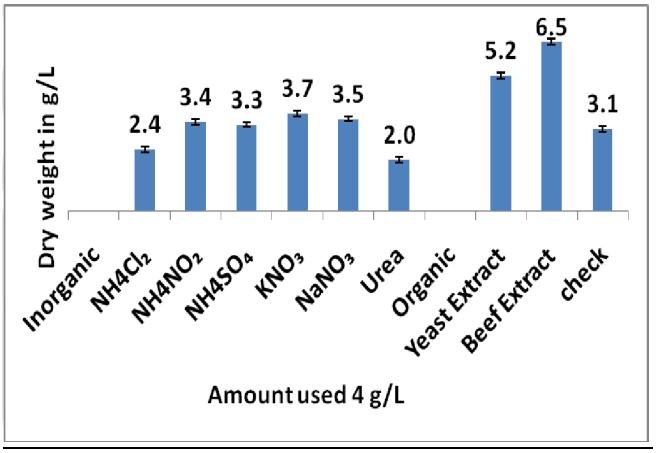

Figure 2: Dry weight obtained by using different nitrogen sources

c. Vitamins

The mycelial yield with folic acid was the highest among the tested vitamins (Table & Fig 3). Mycelial growth was least with pyridoxine, but no significant difference was observed among thimine and nicotinic acid. Growth in D- Biotin and riboflavin were next to folic acid.

Table 3: Effect of Vitamin sources on mycelium growth of *O. sinensis*

Vitamins	Amount used (mg/L)	Dry weight (g/L)
Folic acid	10	8.7 a
D-Biotin	10	7.3 b
Thiamine	10	6.5 c
Nicotinic acid	10	5.7 d
Riboflavin	10	4.5 e
Pyridoxine	10	4.2 e
Check (No vitamin)	-	3.6 f

(LSD at 5% is 0.3 and SE 0.1)

The mean Values indicated by different letters are significantly different by LSD test ($p<0.05$)

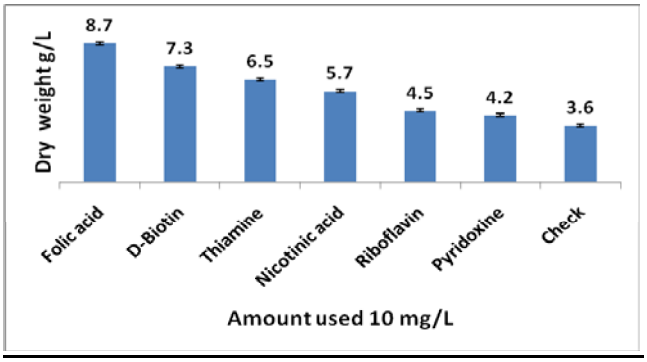

Figure 3: Dry weight obtained by using different vitamin sources

d. Macro (elements) Minerals

The mycelial growth of *O. sinensis* was maximum in $CaCl_2.2H_2O$ and then in NaCl (Table & fig 4). The least growth was oberved in $MgSO_4.7H_2O$. There was a very little difference in dry mycelium weight in KH_2PO_4 and K_2HPO_4, but statistically there was a significant difference.

Table 4: Effect of Macro Minerals on mycelium growth of *O. sinensis*

Minerals	*Amount (g/L)*	*Dry weight (g/L)*
Macro		
$CaCl_2.2H_2O$	1	5.8 a
KH_2PO_4	1	4.6 c
K_2HPO_4	1	4.5 c
$MgSO_4.7H_2O$	1	3.8 d
NaCl	1	5.1 b
Check (No minerals)	-	3.5 d

(LSD at 5% is 0.4 and SE 0.1)

The mean Values indicated by different letters are significantly different by LSD test ($p<0.05$)

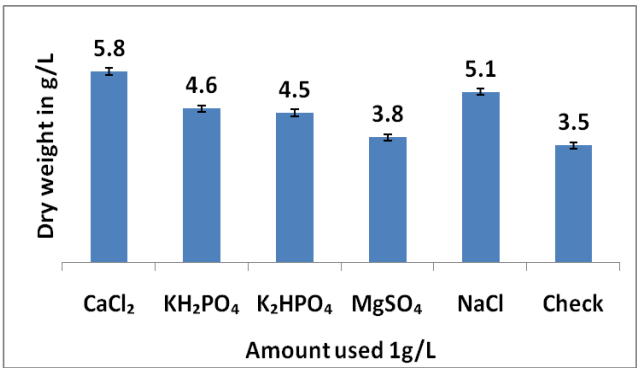

Figure 4: Dry weight obtained by using different macro minerals

e. Micro (elements) Minerals

The mycelial growth of *O. sinensis* was significantly highest in the medium containing $ZnCl_2$ and lowest in $FeSO_4$ (Table & Fig 5). $7H_2O$. Growth in $MnCl_2$.$4H_2O$ is less then $ZnCl_2$ but more than $FeSO_4$. Growth in $CuSO_4$.$5H_2O$ was next to $ZnCl_2$.

Table 5: Effect of Micro Minerals on mycelium growth of *O. sinensis*

Minerals	*Amount (mg/L)*	*Dry weight (g/L)*
Micro		
$CuSO_4$.$5H_2O$	500	6.2 b
$FeSO_4$. $7H_2O$	500	5.1 c
$MnCl_2$.$4H_2O$	500	6.1 b
$ZnCl_2$	500	7.0 a
Check (No minerals)	-	3.4 d

(LSD at 5% is 0.3 and SE 0.1)
The mean Values indicated by different letters are significantly different by LSD test ($p<0.05$)

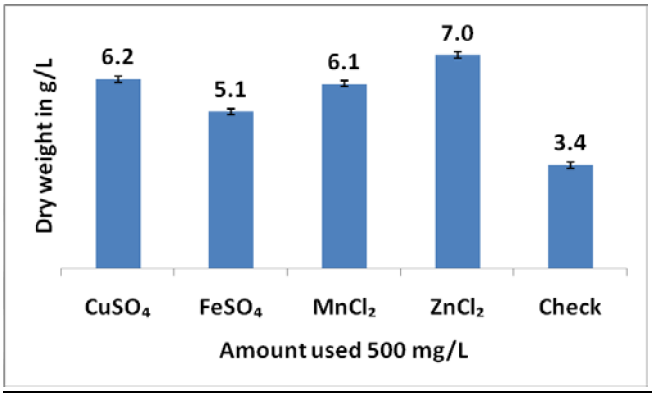

Figure 5: Dry weight obtained by using different micro sources

f. Optimized media

The dry weight obtained from optimized media was significantly higher (12.08g/L) than that of PD broth (3.85 g/L) media used. So this optimized media (Table & Fig 6) can facilitate the research for mass production of this fungus and can be use for large scale industrial production of this fungus.

Table 6: Effect of mycelium growth on optimized medium

S. No.	Optimized medium						*Dry Wt (g /L)*	*Ave dry Wt (g/L)*
	PD broth (g/L)	*Sucrose (g/L)*	*Beef extract (g/L)*	*Folic acid (mg/L)*	*$CaCl_2.2H_2O$ (g/L)*	*$ZnCl_2$ (mg/L)*		
1.	24	30	4	10	1	500	11.75	
2.	24	30	4	10	1	500	12.15	12.08
3.	24	30	4	10	1	500	12.35	

Table7: Effect of mycelium growth on PD broth without optimizes medium

Sl. No.	*PD broth (g/L)*	*Dry Wt (g/L)*	*Ave dry Wt(g/L)*
1.	24	3.78	3.85
2.	24	3.65	
3.	24	4.12	

The distribution of *O. sinensis* at high altitude emphasizes the cool-loving feature of the species and the difficulty in obtaining its living culture from high altitude areas are the main two obstacles to the study of this fungus. During the laboratory culture of this fungus a suitable medium with proper nutritional requirements is essential. Among all, carbon sources (including glucose, maltose, fructose and sucrose), sucrose have been reported for the maximum mycelial growth. With regard to the cost and feasibility in

handling, maltose and sucrose are considered as more suitable substrate.

The most widely used nitrogen sources for mycelia growth are ammonia, ammonium salts, amino acids and complex organic nitrogen **[29]**. As far as nitrogen nutrition is concerned, *O. sinensis* has a greater preference for organic nitrogen, which is common in fungi. For nitrates (potassium and sodium nitrates) and ammoniums (ammonium chloride and ammonium sulphate), it seems that *O. sinensis* prefers the former.

Vitamins produce a growth response at very low concentrations and typically have a catalytic function in the cell as coenzymes or constituents of coenzymes **[29]**. Folic acid and biotin were found to be most suitable for increasing the mycelial growth. Some researcher reported that biotin also enhances the production of citric acid (66.4%) by Aspergillus niger **[30].**

Ophiocordyceps sinensis is more auxoheterotrophic for folic acid than for any other vitamin sources. Derivatives of folic acid are important coenzyme involved in the transfer of one carbon unit which is the material for the synthesis of nucleic acids and proteins **[28].**

Among the macro-elements, the highest mycelial yield of *O. sinensis* was obtained on the calcium and sodium chloride. Chloride form is more suitale then phosphate. Among the trace-elements, the highest mycelial yield of *O. sinensis* was obtained on the medium with zinc. It was also observed that zinc was indispensable for the production of rubratoxin B by *Penicillum rubrum* Sopp and can increase the mycelia dry weight twofold or three fold **[31].** Zinc is a functional component of a variety of fungal enzymes ranging from those involved in intermediary metabolism to the synthesis of DNA and RNA **[29]**.

4. Conclusion

By using this optimized synthetic media, the effect of nutritional requirements of mycelial growth in *O. sinensis* can be studied in detail. The optimized nutritional requirements designed in this study are helpful for the mycelial growth in *O. sinensis.* Further optimization is required to achieve the demands of large-scale mycelial production, which is an ongoing project in this laboratory.

5. Acknowledgments

The authors are grateful to the Director, DIBER, and Haldwani for providing all facilities to carry out the present work. DG, DRDO is thankfully acknowledged to award Fellowship to Ms Seema Singh.

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