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Nutritional Requirements for Mycelial Growth of *Ophiocordyceps sinensis* on Agar Media

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Abstract

Ophiocordyceps sinensis has been used as one of the most valuable traditional Chinese Medication. This fungus parasitizes larva of *Hepialus armoricanus*, and converts each larva into a sclerotium form, in which the fruit body grows. Due to the geographical limitation, where *O. sinensis* can only be found in Himalayas region, the natural resources are limited and very expensive. This research aims to compare the growth-rate of *O. sinensis* mycelia with different ingredients mix with agar media using one-factor-at-a-time method. This research demonstrated the mycelial growth-rate with different carbon sources, including monosaccharide (Fructose, Glucose), disaccharide (Maltose, Sucrose), and polysaccharide (Dextrin, Malt extract), complex organic nitrogen sources, including beef extract, yeast extract, whey protein, and soy protein, and eight different carbon to nitrogen ratios. The objective of this research is to find out the suitable carbon and organic complex nitrogen sources and ratio for the *O. sinensis* solid cultivation. As results, *O. sinensis* grew best with disaccharides comparing to the other types of carbon sources. Furthermore, *O. sinensis* preferred whey protein in contrast to other organic complex nitrogen sources. As for the carbon to nitrogen ratios, an optimal ratio of 18:1 was observed. Based on those experiments, carbon source shows a greater influence for the mycelial growth. Hence many different types of grains and cereals would be great candidates as the main ingredients for the *O. sinensis* solid cultivation.

Keywords

Ophiocordyceps sinensis, Solid Cultivation, Disaccharides, Whey Protein, C/N Ratios, Grains

1. Introduction

Ophiocordyceps sinensis has been used as one of the most valuable traditional

Chinese medicines since the ancient time. In 2015, *O. sinensis* cost as much as 60,000 U.S. dollar per kilogram [1]. *O. sinensis* has a unique way of life and earns this caterpillar fungus a name in Chinese called "Dong Chong Xia Cao", which roughly describes the complexity of the fungus' life cycle [2]. The life cycle of *O. sinensis* includes an asexual and a sexual phase. This fungus parasitizes larva of *Hepialus armoricanus*, and converts each larva into a sclerotium, where the stroma and fruiting body may form under appropriate conditions [3] [4] [5] [6].

O. sinensis has been used as a medicinal herb in traditional Chinese medication to cure chronic lung issues and kidney infections in China for hundreds of years [7]. Nowadays, researches had discovered that *O. sinensis* consists of many active constituents that can be beneficial for human health, for instance mannitol, nucleosides, ergosterol, cordycepin, cordycepic acid and so on; which can be used in treatments related to antitumor effects, organ-transplantation, and the prevention of kidney, liver, and heart diseases [8] [9]. Due to the geographical limitation where *O. sinensis* can only be found at the high altitudes environment within the Himalayas region, the natural *O. sinensis* products are limited and expensive [10] [11]. In the recent years, the price of the natural *O. sinensis* has continuously increased and gradually become a main income source for many local residents. Consequentially, *O. sinensis* is now under threat due to the rapid deforestation, climate change, and overharvesting that is taking place in China, India, and Nepal. More than 40 percent of the *O. sinensis* natural habitats are facing desertification; meanwhile the amount of *O. sinensis* harvested has increased three to five percent annually [12] [13]. Therefore, developing a new solid cultivation media for *O. sinensis* to grow is viewed as a promising alternative for the natural products.

In order to create the most suitable cultivation conditions for *O. sinensis* fungus to grow in the solid media, identifying the preferable mixture of nutrients for the fungus is an essential step to begin. The objective is to find the suitable carbon sources, organic complex nitrogen sources, and carbon to nitrogen ratio by comparing the growth-rate of *O. sinensis* mycelia with different ingredients in agar media using one-factor-at-a-time method.

2. Materials and Methods

2.1. Fungal Strains

The fungal strain used in this study was obtained from the mushroom culture bank at the Laboratory of Forest Production Control, Kyushu University. It is assigned to accession number KUMB108 in the culture bank. The strain was originally brought from Jilin Agricultural University, China. After genetic analysis its internal transcribed spacer (ITS) sequence was found to be an exact match (100%) of NCBI Gene bank entry EF555097.3 *O. sinensis* strain Ph-4Qinghai. In the NCBI Gene bank this strain had been recorded as *O. sinensis* Ph-4Qinghai before being updated to *Paecilomyces hepiali* strain Ph-4Qinghai.

2.2. Carbon Sources

In order to determine the most suitable carbon sources, there are six types of carbon sources were tested including two types of monosaccharide (Fructose, Glucose), two types of disaccharide (Maltose, Sucrose), and two types of polysaccharide (Dextrin, Malt extract) and control. Eight grams of each carbon source was added individually to the base medium, which obtaining 9.8 g of agar and 250 ml of distilled water. After the tested subjects preparations were completed, tested subjects were autoclaved at 121˚C for 30 minutes. Then tested subjects were poured into 15 petri dishes when the subjects were cooled to 50˚C room temperature. Each petri dish was inoculated at the center with a five mm diameter agar plug containing actively growing mycelia of *O. sinensis*. Then all subjects were incubated in the incubator with the temperature setting at 21˚C constant with humidity setting at 60%. All subjects were observed after 21 days after inoculation. Three repetitions were carried out for all six different types of carbon sources with the total of 45 dishes per carbon source.

2.3. Organic Complex Nitrogen

Based on the previous researches, organic complex nitrogen sources showed a more favorable result of the mycelial growth-rate of *O. sinensis* comparing to inorganic nitrogen and amino acids [14]. Therefore, four types of organic complex nitrogen sources were tested including beef extract, yeast extract, whey protein, soy protein, and control (no nitrogen). Eight grams of each nitrogen source was added individually to the base medium, which containing 9.8 g of Potato Dextrose Agar (PDA) and 250 ml of distilled water. After the tested subjects preparations were completed, tested subjects were autoclaved at 121˚C for 30 minutes. The tested subjects were poured into 15 petri dishes when the subjects were cooled to 50˚C room temperature. Each petri dish was inoculated at the center with a five mm diameter agar plug containing actively growing mycelia of *O. sinensis*. Then all subjects were incubated in the incubator with the temperature setting at 21˚C and humidity setting at 60%. All subjects were observed after 21 days after inoculation. Three repetitions were carried out for all six different types of carbon sources with the total of 45 dishes per organic complex nitrogen source.

2.4. Carbon to Nitrogen Ratio

Sucrose and peptone were used for this experiment for carbon to nitrogen ratios. Eight different types of carbon to nitrogen ratios with the same total organic matter of 7.6 g were prepared, shown in **Table 1**. Then each bottles was added four grams of agar and 200 ml distilled water. After the tested subjects preparations were completed, tested subjects were autoclaved at 121˚C for 30 minutes. Tested subjects were poured into 12 petri dishes. Each petri dish was inoculated at the center with a five mm diameter agar plug containing actively growing mycelia of *O. sinensis*. Then all subjects were incubated in the incubator with the

Table 1. Chemical composition of the eight carbon to nirtogen ratios.

C:N Ratio	Source (g)	C %	Peptone (g)	N%	Total Organic Matter
1:1	1.754	0.702	5.846	0.702	7.60
6:1	4.885	1.954	2.714	0.326	7.60
12:1	6.000	2.400	1.600	0.192	7.60
18:1	6.414	2.566	1.186	0.142	7.60
24:1	6.674	2.670	0.930	0.112	7.60
36:1	6.956	2.782	0.644	0.077	7.60
48:1	7.108	2.843	0.49	0.059	7.60
96:1	7.344	2.938	0.256	0.031	7.60

temperature setting at 21˚C and humidity setting at 60%. All subjects were observed after 21 days after inoculation. Three repetitions were carried out for all six different types of carbon sources with the total of 36 dishes per ratio.

2.5. Determination Method

All *O. sinensis* mycelial growth-rates were determined using its average diameter and CIE whiteness index; expect carbon to nitrogen ratios experiment was only observed using the average diameter. The CIE Whiteness and Color Meter PCE-CSM 1 from PCE Instruments were used to determine the whiteness index. The purpose of using whiteness index is to determine the density of the mycelia growth. Whiteness is determined as a single number index referencing the relative degree of whiteness. The index has been devised that the higher the whiteness, the whiter the material [15]. For the average diameter, the purpose is to detect the rate of the mycelia expanding its colonization. All the petri dishes were draw two lines right after inoculation and using these two lines to calculate the average in order to determine the diameter of the dish.

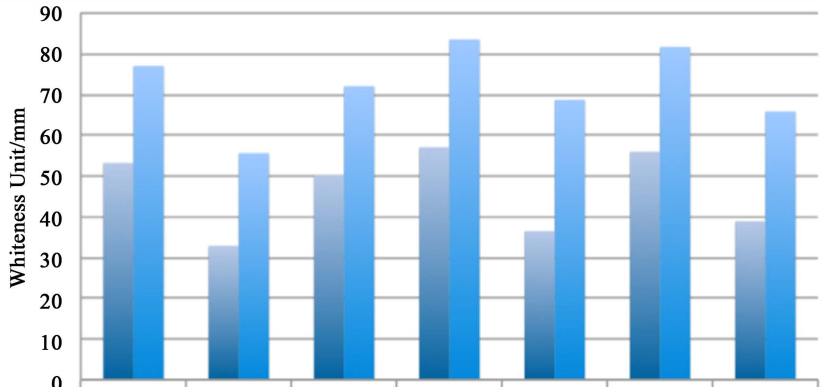
2.6. Statistical Analyses

Identification of statistical differences within treatments was done by Analysis of Variance (ANOVA), followed by Tukey's post hoc test. All the analyses were done with 0.05 significance levels. The analyses were done using Minitab 17 statistical software (Minitab Inc.) and Microsoft Excel.

3. Results

3.1. Carbon Sources

The results of the six different types of carbon sources were shown in **Figure 1**. The highest mycelial growth-rates were obtained by maltose followed by sucrose and dextrin with the colony diameter and whiteness index of 83.56 mm/57.06, 81.73 mm/55.97, and 77 mm/53.18. In contracts, fructose showed a negative effect compared to the control the colony diameter and whiteness index of 55.6



	Dextrin	Fructose	Glucose	Maltose	Malt Extract	Sucrose	Control
Whiteness	53.183	32.830	50.241	57.055	36.458	55.971	38.870
Diameter	77.007	55.601	72.023	83.563	68.691	81.727	65.830

Figure 1. Results of different carbon sources.

mm/32.8. Also malt extract had a positive result on the average whiteness but a negative result of the whiteness comparing to the control.

3.2. Organic Complex Nitrogen Sources

As the complex organic nitrogen sources, whey protein had highest growth to the mycelia growth with the colony diameter and whiteness index of 81.16 mm/48.77, shown in Figure 2. Moreover, Soy protein and yeast extract showed to have a higher whiteness but a slightly lower result of the colony diameter comparing to the control. Beef extract showed a negative influence to the mycelia of *O. sinensis* with the diameter and whiteness of 62.04 mm/36.03 comparing to the control.

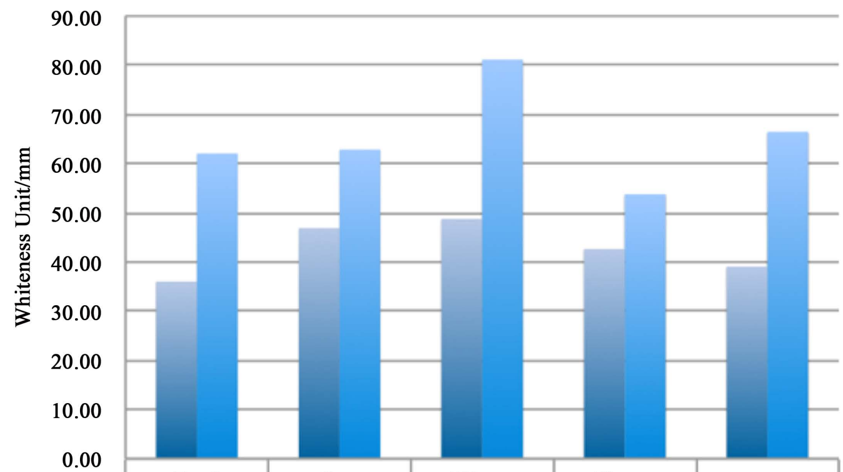
3.3. Carbon to Nitrogen Ratio

An optimal carbon and nitrogen ratio of 18:1 was observed and very high or low ratios resulted in a relatively lower growth rate of the mycelia, shown in Figure 3. However, 6:1, 12:1, and 24:1 did not have a compelling differences compared to 18:1. Carbon sources appeal to have a better influence on the mycelia growth of *O. sinensis* than nitrogen sources.

4. Discussion

Based on the physical and chemical property of the cultivation media, two types of cultivation methods can be performed to cultivate *O. sinensis*. First cultivation method is to use the liquid-state cultivation and the other type of method is using the solid-state cultivation [16]. However, on the industrial level, cultivation production for the fruiting bodies has not been developed successfully in both cultivation methods and the production of the fermented mycelia is only limited to liquid cultivation [17] [18].

Therefore, this research has identified the two main nutrient ingredients for



	Beef Extract	Soy Protein	Whey Protein	Yeast Extract	Control
Whiteness	36.03	46.89	48.77	42.65	39.08
Diameter	62.04	62.87	81.16	53.78	66.46

Figure 2. Results of different organic complex nitrogen sources.

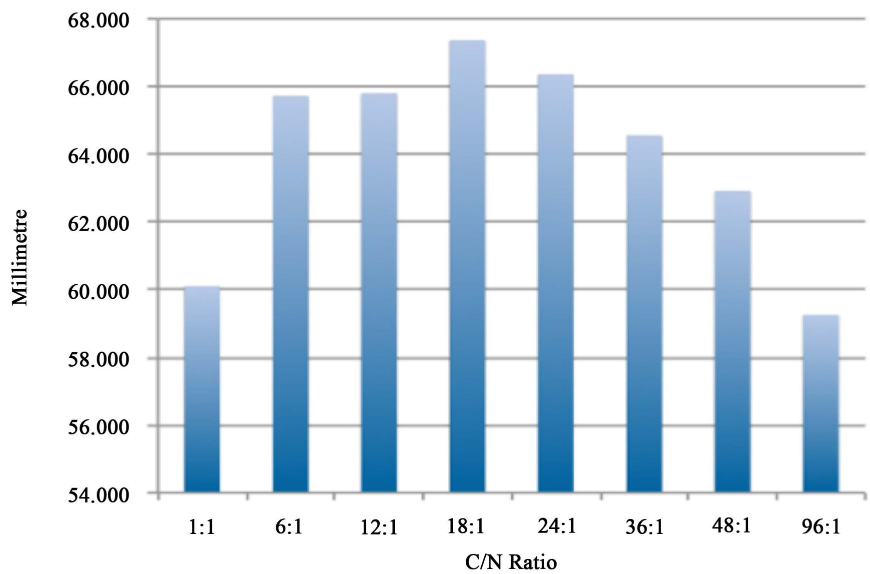


Figure 3. Results of different carbon to nitrogen ratios.

the *O. sinensis*. As this research demonstrated, *O. sinensis*' mycelia grew best with disaccharides (Maltose, Sucrose) in compare to other types of carbon sources. Furthermore, whey protein showed the highest growth-rate followed by soy protein and the optimal carbon to nitrogen ratio of 18:1 was observed.

Based on the researches done in 2013, disaccharide has been shown to be a preferable ingredient for the *O. sinensis* and additional organic nitrogen in the media would enhance the fruiting body yield [14]. Different types of grains and cereals mixture would be potential candidates for the media. Rice has been used commercially as a main ingredient for cultivating *Cordyceps militaris*, which is another types of Cordyceps species [19]. All Cordyceps species have similar life cycles and have developed mechanisms to invade insects and grow on them; the major difference is being the locality where they grow and the host insect they infect [20]. Therefore, to identify different types of grains and cereals mix would be a good candidate as base ingredients for the *O. sinensis* solid cultivation, for example soybean and wheat mix. Soybean contains a lot of glucose, sucrose, and soy protein and wheat has high content of maltose.

Nonetheless, further researches are still needed to find the best ratios and combination of grains mixture in order to reach the optimal fruiting body yield. Furthermore, researches to find the best temperature, humidity of the growth environment, stimulation techniques and so on will also be needed for cultivating *O. sinensis* fruiting body.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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