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Nutritional studies on submerged culture of *Ganoderma lucidum*

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Abstract

In this study nutritional requirements for mycelial growth and polysaccharide formation in submerged culture of *Ganoderma lucidum* were determined. The results indicate that glucose was the best carbon source for mycelial growth and it's concentration ranging from 2 to 5 % caused no much difference in the level of biomass produced. Concerning the effect of C/N ratio, higher yields of mycelial growth were obtained in the range of 30:1 to 60:1 in glucose-ammonium chloride synthetic medium. On the contrary, higher C/N ratio favored the formation of polysaccharide. The presence of insoluble particles such as rice bran or wheat bran in the medium was very helpful to initiate pelleting.

Key words: *Ganoderma lucidum*, submerged culture, mycelial growth, polysaccharide

Introduction

Ganoderma lucidum (Fr.) Krast (Polyporaceae) and related species are fungi used in traditional Chinese medicine. Its fruiting body is called "Reishi" or "Mannentake" in Japanese and "Lingzhi" in China. In the regions of China, Japan, Korea and Taiwan, Lingzhi has been a popular folk or oriental medicine to cure various human diseases, such as hepatitis, hypertension, hypercholesterolemia and gastric cancer. Recent studies on this fungus have demonstrated many interesting biological activities, including antitumour, anti-inflammatory and cytotoxicity to hepatoma cells. These studies also suggested that the carcinostatic substance in Lingzhi is a polysaccharide, β-(1->3) -D-glucan (Mizuno et al., 1995; Sone et al., 1985). This polysaccharide seems to have promise as a new type of carcinostatic agent which might be useful in

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immmunotherapy. Material from *Ganoderma lucidum* showed high activity at a dosage of 10 mg/kg against Sarcoma 180 tumour in mice (Sutherland, 1990). Antitumour activity depends on the source of the polysaccharide. Unlike chemicals used in chemotherapy, it has few toxic side effects.

Lingzhi, because of its perceived health benefits, has gained wide popularity as a health food, in both Japan and China. The 1988 production of Reishi in Japan was estimated to be about 250 tons dry weight. Lingzhi cultivation has also prospered in China, Taiwan, Korea, and Thailand (Mizuno et al. 1995). Because it usually takes several months to culture the fruiting body of Lingzhi, many attempts are being made to obtain useful cellular materials or to produce effective substances from cultured mycelia (Tseng et al., 1984; Sone et al., 1985).

Mushroom mycelia or spawns have normally been produced in solid cultures using substrates such as grain, sawdust or wood. Propagation of edible mushrooms in submerged culture was initially developed during the 1950s based on the success of growing lower fungi in fermenters for economical production of various natural products. Since then numerous attempts have been made by researchers to cultivate mushroom mycelium commercially in submerged culture (Litchfield, 1967). Submerged culture has the potential advantage in that it can be dispersed within the substrate more uniformly than solid spawn and the time taken to produce the first crop of sporophores may be shortened. Further, the liquid nature of such spawn enables inoculation to be carried out under relatively more stringent aseptic conditions which is important when using non-selective substrates (Song and Cho, 1987).

Control of the composition of the fermentation medium, its addition and other environmental parameters are critical in achieving the desired rates of synthesis and yields of microbial products. Although many workers have attempted to obtain mycelium of *Ganoderma lucidum* using submerged culture, very little information is available regarding the environmental factors affecting mycelial growth of *G. lucidum* in submerged culture (Sone et al., 1985; Tseng et al., 1984). The study reported here was carried out to determine the nutritional requirements of *Ganoderma lucidum* for mycelial growth and polysaccharide production (Hwang, 1996).

Materials and Methods

Maintenance of culture

Organism *Ganoderma lucidum* CCRC36123 was obtained from the Culture Collection and Research Center (CCRC), Food Industry Research and Development Institute (Hsinchu, Taiwan). The strain was maintained on potato-dextrose-agar slant. Slopes were inoculated and incubated at room temperature for 7 days, and stored at 4 °C.

Media

The media were made up of the following components: yeast extract 0.1%, K_2HPO_4 0.05%, KH_2PO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05% and other specified carbon sources and nitrogen sources. The C/N ratio of the medium was adjusted by altering the glucose concentration and keeping the concentration of nitrogen constant or by modifying the nitrogen concentration at constant glucose concentration.

Cultivation

The shake-flask experiments were performed in 500-ml Erlenmeyer flasks containing 100 ml of the media. Media were sterilized at 120 °C for 20 min and glucose was autoclaved separately. The pH was measured and adjusted to the desired value by addition of either 4M-HCl or 2.5M NaOH. Actively growing mycelia from a newly prepared slant culture (about 7 days incubation at 30 °C) were inoculated into the flask. The flasks were shaken on a displacement shaker (Model 903, Hotech Co.) at 100 rpm and 30 °C. At the end of incubation period mycelium consisting of individual pellets was harvested by centrifugation and wash for the analysis. The yield was expressed as mg/100ml dry weight (Hwang, 1996).

Analytical methods

The pH was measured with a digital pH meter (Suntex, Taiwan, model 2000A). Due to the fact that mycelia and cell-bound polysaccharide could not be thoroughly separated by centrifugation, in order to determine the concentrations of mycelium and polysaccharide, samples were first subjected to ultrasonication for 2 hrs in a Branson ultrosonicator (model 5210). Centrifugation was then performed to remove cells and cell debris in a centrifuge (Hettich, model

ERA3S/10ml). Dry weights of total cell mass were obtained by centrifuging samples at 3000 rpm for 10 min, washing the sediment three times with water, and drying to constant weight. All supernatants were collected, and then the crude polysaccharide was precipitated with the addition of 4 volumes of 95% ethanol. The precipitated polysaccharide was collected by centrifugation at 3000 rpm for 10 min and then dried to remove residual ethanol at 60 °C. Total polysaccharide in the culture medium was determined by the method of phenol-sulfuric acid assay according to Dubois et al.(Dubois et al., 1956; Pazur, 1987).

Results and Discussion

Effect of glucose concentration

According to Litchfield in 1979, the concentration of purified carbohydrate suitable for production of fungal mycelium is in the range of 1 to 10%. In this study, concentration of malt extract was fixed at 4 % to investigate the effect of glucose concentration on mycelium growth and polysaccharide production. The results are presented in Table 1. It was found that the concentrations of glucose ranging from 2 to 5 % caused no much difference in the level of biomass produced. High concentration of carbohydrate and nitrogen sources are usually needed in order to achieve a high yield (dry weight) of mycelium. But too high a concentration of glucose can have an inhibitory growth effect on several mushrooms. Therefore a growth medium should be optimized in order to obtain low cost (Eyal, 1991). In this test malt extract was found to interfere with the determination of polysaccharide and was not used in the following tests.

Table 1. Effect of glucose concentration on the production of mycelium and polysaccharide

Glucose conc. (%)	Mycelium conc. (mg/100ml)	Polysaccharide conc. (mg/100ml)	Final pH
2	378	73	4.3
3	349	83	4.2
5	369	55	4.5

1. under the conditions of initial pH=5.65, 30°C and 100 rpm for 7 days

2. malt extract 4%, yeast extract 0.1%, K_2HPO_4 0.05%, KH_2PO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%.

Effect of different carbohydrates

The results shown in Table 2 reveal the suitability of different carbohydrate for the growth of *G. lucidum* mycelium and polysaccharide production. Glucose was the best carbon source for mycelial growth and this agrees with the general concept that, among all the hexoses, glucose is the biologically the most effective energy source. However, sucrose seemed to be in favor of the formation of polysaccharide.

Table 2. Effect of different carbon sources on the production of mycelium and polysaccharide

C-source	Mycelium conc. (mg/100ml)	Polysaccharide conc. (mg/100ml)	Final pH
Sucrose	138.6	14.26	3.01
Fructose	140.0	5.28	4.01
Manitol	108.6	3.80	4.04
Sorbitol	101.0	4.70	3.88
Glucose	230.0	9.40	3.50

1. under the conditions of initial pH=5.65, 30°C and 100 rpm for 7 days

2. C-source 3%, $NH_4H_2PO_4$ 0.123% (C/N=80), yeast extract 0.1%, K_2HPO_4 0.05%, KH_2PO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%.

Effect of C/N ratio

C/N ratio---- According to the experience obtained from submerged culture of mushroom mycelium, the range of carbon: nitrogen ratio is very important. This ratio influences the yield and efficiency of the production of mushroom mycelium. Mycelia of many mushrooms will grow to some extent over a wide range of carbon to nitrogen ratios (C/N) in the medium, providing all other nutritional requirements are met; however, highest yields are obtained in a rather narrow range (Litchfield, 1967). In order to produce more mushroom mycelium, the C/N values reported by different investigators to give highest yields also vary widely depending upon species, growth medium, and conditions used. The effects of C/N ratio on mycelial growth and polysaccharide formation in the culture of *Ganoderma lucidum* are shown in Table 3. It can be seen that higher yields of mycelial growth were obtained in the range of 30:1 to 60:1 in glucose-ammonium chloride synthetic medium.

Concerning the effects of C/N ratio on the polysaccharide production, many studies for the production of microbial polysaccharides have been referred and demonstrated that the C/N ratio has a pronounced effect on the polysaccharide production (Margaritis and Pace, 1985). The results in Table 3 show that in contrast to mycelial growth, higher C/N ratio was believed to favor the formation of polysaccharide. The final amount of polysaccharide was negatively affected if the C/N ratio was decreased below 60 at a fixed glucose concentration of 30 g/liter. Similar effects have been observed with other polysaccharide-producing organisms (Margaritis and Pace, 1985).

Table 3. Effect of C/N ratio on the production of mycelium and polysaccharide

C/N ratio	Mycelium conc. (mg/100ml)	Polysaccharide conc. (mg/100ml)	Final pH
10	162.8	4.17	4.10
30	179.1	4.79	3.88
60	179.3	4.31	4.12
80	157.3	5.29	3.57

1. under the conditions of initial pH=5.65, 30°C and 100 rpm for 7 days
2. Glucose 3%, yeast extract 0.1%, K_2HPO_4 0.05%, KH_2PO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%.

Effect of N-sources

In order to produce more polysaccharide, on the basis of the results of the C/N ratio study, the effects of different nitrogen sources on submerged culture of *G. lucidum* were carried out at the fixed C/N ratio of 80. The mycelia of *G. lucidum* that have been grown in submerged culture utilized nitrogen sources including ammonium chloride, ammonium sulfate, ammonium dihydrogen phosphate, ammonium nitrate, sodium nitrate, and sodium glutamate. The results are shown in Table 4 and the addition of ammonium dihydrogen phosphate seemed to be beneficial to both mycelium growth and polysaccharide formation. This could be attributed to the presence of phosphorous.

Table 4. Effect of various N-sources on the production of mycelium and polysaccharide

N-source	Mycelium conc. (mg/100ml)	Polysaccharide conc. (mg/100ml)	Final pH
NH_4NO_3	193.3	6.91	4.29
$NaNO_3$	229.1	6.38	4.58
$(NH_4)_2SO_4$	208.3	6.35	3.57
$NH_4H_2PO_4$	230.9	9.35	3.50
NH_4Cl	157.3	5.29	3.57
Sodium glutamate	208.2	7.59	4.61

1. under the conditions of initial pH=5.65, 30°C and 100 rpm for 7 days
2. Glucose 3%, yeast extract 0.1%, K_2HPO_4 0.05%, KH_2PO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%.
3. C/N=80

Effect of rice bran and wheat bran

Previous works have pointed out the need for using insoluble supports to initiate pelleting (Martin and Bailey, 1985). When rice bran was homogenized in a blender and used as the nitrogen source, pellets formed very rapidly in the second day. The insoluble was encapsulated first by the mycelia, and decomposed gradually and nearly disappeared at last. Owing to containing some insolubles in the media, the data for mycelium concentration could not obtain easily. However, the results observed by naked eyes indicate that the presence of solid particles in the fermentation could be very helpful to initiate pelleting. The utilization of rice bran and wheat bran as N-sources deserve further study.

In summary, the results obtained from this study indicate that many nutritional factors could affect mycelia growth and polysaccharide formation in submerged culture of *Ganoderma lucidum*. As described above, control of the composition of the fermentation medium, its addition and other environmental parameters are critical in achieving the desired yields of microbial products. Although some researcher reported that the formation of polysaccharide in submerged culture of *Ganoderma lucidum* was attributed to primary metabolite (Lee, 1989), it was not proved by this research. The optimal conditions for mycelial growth should be different from those for polysaccharide formation.

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靈芝液體培養之營養需求

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摘 要

本研究針對靈芝液體培養時，不同營養成份對菌絲體及多醣體生成之影響進行探討。對菌絲生長而言，葡萄糖爲良好碳源，而葡萄糖濃度2至5%對菌絲生成無差別影響；相對地，以蔗糖爲碳源，有利於多醣體生成。碳源與氮源比值在30:1至60:1範圍時，菌絲體生長較佳；而更高的碳源與氮源比值有利於多醣體生成。當培養基中含有米糠或麩皮時，將有助於顆粒形成。

關鍵詞: 靈芝；液體培養；菌絲體生長；多醣體

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