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Stimulatory effects of *Coix lacryma-jobi* oil on the mycelial growth and metabolites biosynthesis by the submerged culture of *Ganoderma lucidum*

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ABSTRACT

Effects of *Coix lacryma-jobi* oil (CLO) addition on the mycelia growth and production of bioactive metabolites, such as triterpenoids, exopolysaccharide (EPS), and intracellular polysaccharide (IPS) in the submerged culture of *Ganoderma lucidum* were studied. The results showed that when a level of 2% CLO was added at the beginning of culture, the biomass, triterpenoids, EPS, and IPS productions reached a maximum of 10.71 g/L, 92.94 mg/L, 0.33 g/L, and 0.389 g/L, respectively, that were 3.34-fold, 2.76-fold, 2.2-fold, and 2.23-fold compared to that of control. Analysis of fermentation kinetics of *G. lucidum* suggested that glucose concentration in the culture of CLO-added group decreased more quickly as compared to the control group from day 2 to day 7 of fermentation process, while the triterpenoids and polysaccharides biosynthesis were promoted at the same culture period. However, the culture pH profile was not affected by the addition of CLO. There were no new components in the two types of polysaccharides obtained by the addition of CLO. Enzyme activities analysis indicated CLO or its fatty acids affected the synthesis level of phosphoglucose isomerase and α-phosphoglucomutase at different stage.

1. Introduction

Medicinal mushrooms have long been used in traditional oriental therapies, and modern scientific and medical studies demonstrate the potent and unique properties of mushroom-extracted compounds for the prevention and treatment of cancer [1]. *Ganoderma lucidum* (Fr.) Karst, one of the most popular mushrooms used in traditional Chinese medicine, has been used to prevent and treat various human diseases such as hepatitis, chronic bronchitis, hypertension, hypercholesterolemia, and gastric cancer for more than thousand years [2]. Pharmaceutically active compounds from fruiting body and mycelium of *G. lucidum* include polysaccharides, proteins, triterpenoids, proteopolysaccharides, sterols, alkaloids, and nucleotides [3,4]. Among them, polysaccharides and triterpenoids are the two main functional components that are considered to possess multiple biological activities for therapeutic use [5].

Because of its perceived nutritional and health values, *G. lucidum* has gained wide popularity as a nutraceuticals and functional foods in China, Japan, Korea and other regions. Normally, *G. lucidum* is available in the form of mature fruiting bodies and spores by solid cultures using substrates such as grain, sawdust or wood. However, the production of fruiting bodies and spores includes a long cultivation for about 6 months [6]. In recent years, submerged culture of *G. lucidum* has been developed because of the potential for higher mycelia and bioactive components production in a compact space and in shorter time with fewer chances for contamination, in which mycelial biomass, triterpenoids, and polysaccharides are the desired products [7,8]. Secondary metabolite production by *G. lucidum* is affected by several factors. To accelerate mycelial growth and metabolite production by *G. lucidum*, the effects of environmental conditions [9], medium composition [10], inoculation density [11], pH [12,13], two-stage culture process [14], oxygen supply [15], pH-shift and DOT-shift integrated fed-batch fermentation [16], etc. have been studied. It is known that designing an appropriate fermentation conditions, besides of a productive strain construction, is crucial for optimization of the microbial fermentation processes [17]. To enhance the production efficiency, modification of media composition would be vital in the submerged culture of *G. lucidum* [6].

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Coix lacryma-jobi, a distant relative of maize in the *Maydeae* tribe of the grass family *Poaceae*, is native to India, Burma, China, and Malaysia and grown extensively in South Asia before maize became popular as an agricultural crop [18]. The seed of *C. Lacryma-jobi* is full of starch, protein, oil, mineral elements, and vitamins, and has been used as a food source for humans and livestock, in the production of alcoholic beverages and as a medicinal plant over the years [18]. Our previous work showed *C. lacryma-jobi* was a good media ingredient and could improve the growth and bioactive metabolites production of *G. lucidum* [19]. It was reported that mycelial growth and metabolite production of *G. lucidum* could be induced by plant oils [20], The objective of this work was to examine the effects of *C. lacryma-jobi* oil (CLO) on the mycelial growth, triterpenoids and polysaccharides production by medicinal mushroom *G. lucidum* in submerged culture, and the influences on polysaccharides components and related biosynthesis enzyme activities were also explored.

2. Materials and methods

2.1. C. lacryma-jobi and C. lacryma-jobi oil (CLO) extraction

The seeds of *C. lacryma-jobi* were purchased from Yetongren Medicinal Co. Ltd. (Wenzhou, China), dried and pound to powder (40 mesh), then stored at 4 °C. CLO extraction was conducted using Soxhlet equipment as follows: in batches, seeds (50 g) were extracted with hexane (500 mL) for 5 h, and the solvent was then evaporated.

2.2. Microorganism and culture conditions

G. lucidum WZ06 was screened and collected by the Laboratory of fermentation, Wenzhou University (Wenzhou, People's Republic of China) and maintained on potato dextrose agar (PDA). The inocula were prepared in a 500 mL Erlenmeyer flask containing 150 mL media (see below) at 30 °C for 7 days with shaking at 160 rpm. This was then inoculated at 10% (v/v) into the same medium now containing different amount of CLO. The cultivation medium contained (g/L): glucose, 35; peptone, 3.5; KH_2PO_4, 1.0; K_2HPO_4, 1.0; $MgSO_4$, 0.75; and vitamin B_1, 0.01.

2.3. Determination of biomass

Biomass was obtained by centrifuging at 8000 rpm for 20 min, washing the precipitated cells for three times with distilled water, and drying at 60 °C for a sufficient time to a constant weight.

2.4. Measurements of extracellular and intracellular polysaccharides

For the determination of exopolysaccharides (EPS), after the removal of mycelia by centrifugation, the crude polysaccharide was precipitated with adding 4 times of 95% (v/v) ethanol. The precipitated polysaccharide was collected by centrifugation at 8000 rpm for 20 min, and washed with 80% (v/v) ethanol three times, then dried to remove residual ethanol at 60 °C. Total polysaccharide in the culture medium was determined by the phenol-sulfuric acid assay. For the analysis of intracellular polysaccharides (IPS), the dried mycelia were extracted with 1 mol/L NaOH at 60 °C (1 h), and then the supernatant was assayed by phenol–sulfuric acid method [7].

2.5. Assay of triterpenoids

Triterpenoids production was measured as described by Fang and Zhong [13] with some modification. The dried mycelia (100 mg) were extracted by 50% (v/v) ethanol (5 mL) for 1 week (twice). After removal of mycelia by centrifugation, the supernatants were dried at 50 °C under vacuum. The residues were suspended by water, and later extracted with chloroform. The triterpenoids in the chloroform extract were further extracted with 5% (w/v) $NaHCO_3$. The pH of $NaHCO_3$ phase was adjusted to 2.0–3.0 by 3 mol/L HCl at 0 °C, and then the triterpenoids in the $NaHCO_3$ phase were again extracted with chloroform. After removal of chloroform by evaporation, triterpenoids were dissolved in absolute ethanol, and its absorbency was measured at 245 nm in a spectrophotometer (Puxi General Analytical Instrument Factory, Beijing, China).

2.6. Analysis of polysaccharide components

The IPS and EPS Polysaccharides from the samples of containing 2% (v/v) CLO in the media and control were respectively collected as the method mentioned above, and then fractionated on ÄKTA Explorer (Sweden). 2 mL polysaccharide (about 2–2.5 mg/mL) of each sample was eluted on a column (HiPrep 16/10 DEAE) with H_2O and followed stepwise by 0.05, 0.2, and 0.5 mol/L NaCl at 2 mL/min. Fractions (5 mL) were assayed by the phenol-sulfuric acid method. The main components of IPS and EPS were re-fractionated on a column of Superdex 200 HR 10/30 with 0.1 mol/L NaCl at 0.25 mL/min, respectively. Fractions (1 mL) were assayed by the phenol-sulfuric acid method.

2.7. Enzyme assays

Mycelia were harvested by centrifugation at 15,000 × *g* for 30 min, washed twice with 0.9% NaCl, and suspended in 20 mmol/L phosphate buffer (pH 6.5) containing 50 mmol/L NaCl, 10 mmol/L $MgCl_2$, and 1 mmol/L dithiothreitol. Mycelia were disrupted ultrasonically at 0 °C and cell debris was removed by centrifugation. The protein content of the cell extract was determined by the method of Bradford [21]. Enzyme assays were performed at 30 °C in a total volume of 1 mL with freshly prepared cell extracts. The formation or consumption of NAD(P)H was determined by measuring the change in the absorbance at 340 nm as described by Looijesteijn et al. [22].

The α-phosphoglucomutase (EC 2.7.5.1) reaction mixture contained 50 mmol/L triethanolamine buffer (pH 7.2), 5 mmol/L $MgCl_2$, 0.4 mmol/L NADP, 50 μmol/L glucose-1,6-diphosphate, 4 U of glucose-6-phosphate dehydrogenase, and cell extract. The reaction was started by adding 1.4 mmol/L α-glucose-1-phosphate.

The phosphoglucose isomerase (EC 5.3.1.9) reverse reaction mixture contained 50 mmol/L potassium phosphate buffer (pH 6.8), 5 mmol/L $MgCl_2$, 0.4 mmol/L NADP, 4 U of glucose-6-phosphate dehydrogenase, and cell extract. The reaction was started by adding 5 mmol/L fructose-6-phosphate.

The UDP-glucose pyrophosphorylase (EC 2.7.7.9) reverse reaction mixture contained 50 mmol/L Tris–HCl buffer (pH 7.8), 14 mmol/L $MgCl_2$, 0.3 mmol/L NADP, 0.1 mmol/L UDP-glucose, 2.1 U of α-phosphoglucomutase, 4 U of glucose-6-phosphate dehydrogenase, and cell extract. The reaction was started by adding 4 mmol/L inorganic pyrophosphate.

The reaction mixture of the dTDP-glucose pyrophosphorylase (EC 2.7.7.24) reverse reaction mixture contained 50 mmol/L Tris–HCl buffer (pH 7.8), 8 mmol/L $MgCl_2$, 0.3 mmol/L NADP, 0.1 mmol/L TDP-glucose, 2.1 U of α-phosphoglucomutase, 4 U of glucose-6-phosphate dehydrogenase, and cell extract. The reaction was started by adding 4.7 mmol/L inorganic pyrophosphate.

2.8. Statistical analysis

Cultures were performed in a triplicate and data were analyzed by using Statistics Analysis System (SAS) 8.1 version (SAS Institute Inc., USA). The results were expressed as the mean ± SD. The