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Influence of nutritional components and oxygen supply on the mycelial growth and bioactive metabolites production in submerged culture of *Antrodia cinnamomea*

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Abstract

Effects of carbon sources, nitrogen sources, plants oils and oxygen supply on the cell growth and production of bioactive metabolites such as exopolysaccharide (EPS), intracellular polysaccharide (IPS) and triterpenoid in the submerged culture of *Antrodia cinnamomea* CCRC36716 were studied in detail. Malt extract (ME), yeast extract (YE) and corn steep powder (CSP) were favorable nitrogen sources to the mycelial growth. The highest cell growth (12.52 ± 0.03 g/l cell dry weight), EPS production (1861 ± 62 mg/l) and IPS content (41 ± 12 mg/g DW) can be obtained on day 10 of cultivation in the medium containing 3% CSP, 3% YE, and 3% ME, respectively. The highest overall triterpenoid production (30 mg/g DW) was obtained in 3% CSP medium after 14 d of cultivation. Amongst five carbon sources examined, maltose and glucose yielded relatively high mycelial biomass; high cell densities of 8.29 ± 0.05 and 8.69 ± 0.05 g DW cells/l were obtained after 10 d of cultivation when 4% of maltose and glucose was used, respectively. Pronounced production of EPS, 1482 ± 63 and 1318 ± 48 mg/l, was obtained after 10 d of cultivation when 4% of lactose and sucrose was used, respectively. The maximum IPS content (49 ± 9 mg/g DW) was achieved in 4% glucose medium after 10 d of cultivation; the highest overall triterpenoid production (31 mg/g DW) was obtained in 2% glucose medium after 14 d of cultivation. All plant oils tested stimulated cell growth of *A. cinnamomea*, enhanced the production of IPS, but inhibited the triterpenoid production. EPS production was slightly inhibited with soy oil but enhanced by the other oils tested, and the maximal EPS production (1147 ± 47 mg/l) was obtained when 0.5% of peanut oil was supplemented. The high O_2 supply in the *A. cinnamomea* culture was favorable for cell growth and polysaccharide production, but was inhibitory on the triterpenoid production. The results obtained are useful in regulation and optimization of *A. cinnamomea* culture for efficient production of cell mass and bioactive metabolites such as EPS, IPS and tripterpenoids in the submerged culture.

Keywords: *Antrodia cinnamomea*; Exopolysaccharide; Intracellular polysaccharide; Triterpenolids

1. Introduction

Antrodia cinnamomea in the Polyporaceae (Aphyllophorales) is known in Taiwan under the name ''Niu-Chang-Ku'' or ''Jang-Jy''. It grows rarely on the inner cavity wall of endemic evergreen *Cinnamomum kanehirai* Hay, and is well known as an expensive medicinal material. It is commonly used as remedy for food, alcohol, and drug intoxication, diarrhea, abdominal pain, hypertension, skin itching and cancer [1]. In the past, phytochemical investigations have resulted in the isolation of a series of sesquiterpene lactone, polysaccharides, steroids and triterpenoids [2–8]. Nevertheless, systemic studies of the bio-function of the extracted ingredients were not performed until recently. Polysaccharide components extracted from *A. cinnamomea* mycelia have been shown exhibiting an anti-hepatitis B virus surface antigen effect [9], having pronounced anti-tumor effects on both in vitro and in vivo model [10], and displaying strong immunomodulatory and anti-inflammatory effects [10,11]. It has also been shown that triterpenoids extracted from *A. cinnamomea* have anti-cholinergic and anti-serotonergic activities.

The fruiting body of *A. cinnamomea* grows extremely slowly in nature which prohibits the collection of sufficient quantities for extensive use as a drug remedy. In recent years, the fruit bodies of *A. cinnamomea* are expensive because of host

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specificity and rarity in nature, and the failure of artificial cultivation; they are sold for as high as US$ 15,000/kg [1]. Thus, investigators have exerted their efforts to prepare this mushroom from submerged culture in the form of mycelium for use in the formulation of nutraceuticals and functional foods [12]. Submerged culture gives rise to potential advantages of higher mycelial production in a compact space and shorter time with lesser chances of contamination [13,14]. In this study, we investigate the nutritional requirements for submerged culture of *A. cinnamomea*, attempting to obtain optimal submerged culture conditions for mycellial growth, polysaccharides and triterpenoid production by this specie.

2. Materials and methods

2.1. Microorganism and media

A. cinnamomea CCRC36716 used in this study was obtained from the Culture Collection and Research Center (CCRC), Taiwan. The strain *A. cinnamomea* was maintained on potato dextrose agar (PDA) slants. The slant was incubated at 25 °C for 7 days, and then stored at 4 °C.

2.2. Inoculum preparation

PMP medium (2 g glucose, 2 g malt extract and 0.1 g peptone in 100 m of distilled water) for seed culture was prepared; the pH was initially adjusted to 5, followed by autoclaving at 15 psi, 121 °C for 15 min. *A. cinnamomea* was transferred to PMP medium by punching out 0.7 mm diameter agar discs from culture grown on PDA plates; five discs were used to inoculate 100 ml liquid media. The seed culture was grown in a 250 ml Erlenmeyer flask at 25 °C on a rotary shaker incubator at 100 rpm for 7 days [15].

2.3. Flask culture conditions

The flask culture experiments were performed in 250 ml flasks containing 50 ml of PMP medium after inoculating with 10% (v/v) of the seed culture. The culture was incubated at 25 °C on a rotary shaker incubator at 100 rpm, and samples were collected at various intervals from the shake flasks for analyzing biomass dry weight, exo-polysaccharides (EPS), intracellular polysaccharides (IPS) and triterpenoids production.

The effects of factors affecting cell growth and the production of components such as EPS, IPS and triterpenoids by *A. cinnamomea* CCRC36716 were studied using shaking flask culture on rotary incubator shaker (Wisdom 721 SR-Incubator-Shaker) as described above. Effects of carbon sources and nitrogen sources on *A. cinnamomea* culture were studied by substituting various carbon sources such as lactose (L), sucrose (S), maltose (M) and fructose (F) for glucose in fermentation medium, and by substituting nitrogen sources such as corn steep powder (CSP), yeast extract (YE) for malt extract (ME) in a one at a time fashion The concentrations of carbon sources used were from 0.5 to 3%, and nitrogen sources were from 1 to 4%.

Effects of plant oils on *A. cinnamomea* culture were also studied using shake flask culture. Plant oils such as soy, peanut, coconut, sun-flower and olive oils were supplemented, all at volume fractions of 0.1 to 1%, in liquid media (2 g glucose, 2 g CSP and 0.1 g peptone in 100 ml of distilled water) and the culture was cultivated at 25 °C on a rotary shaker incubator at 100 rpm.

2.4. Fermentation in a bioreactor and O_2 supply experiment

The fermentation medium was inoculated with 10% (v/v) of the seed culture and the cultivated was carried out in a 5 l stirred-tank fermenter (Firstec Co., Taiwan). Unless otherwise specified, fermentations were performed under the following conditions: temperature, 25 °C; aeration rate, 1 vvm; agitation speed, 100 rpm; initial pH, 5.0; working volume, 4l.

To study the effect of oxygen supply on *A. cinnamomea* culture, the cultures were all agitated at the same speed (100 rpm), and the aeration rate was set initially at 1 vvm with inlet air controlled at 21% and 30% oxygen during fermentation. The 30% oxygen was derived from mixing 21% oxygen (air) with the 95% oxygen produced by an oxygen generator (EYELA, Model SO-004, Japan).

2.5. Determination of mycelial dry weight

For measurement of mycelial dry weight, the mycelia from a sample were filtered through a mesh with 30 μm pore size and washed with a large amount of distilled water, then collected by filtration through a pre-weighted Whatman filter paper no. 2 (Whatman International Ltd., Maidstone, UK), followed by freeze drying to a constant dry weight.

2.6. Measurements of extracellular and intracellular polysaccharides

Samples collected at various intervals from shake flasks were centrifuged at 6000 rpm for 15 min, and the resulting supernatant was filtered through a Whatman filter paper. The resulting culture filtrate was mixed with four volumes of 95% (v/v) ethanol, stirred vigorously and left overnight at 4 °C. The precipitated EPS was recovered by centrifugation at 6000 rpm for 15 min and the supernatant was discarded. The precipitate of crude EPS was lyophilized and suspended in 1 M NaOH at 60 °C for 1 h, and reducing sugar in the supernatant were measured by phenol-sulfuric acid method [16]. Intracellular polysaccharides (IPS) were extracted from dried mycelia (100 mg) by suspending the mycelia in 10 ml distilled water and autoclaving at 15 psi, 121 °C for 15 min [17]. The amount of IPS was then determined by the procedure used for EPS measurement as described above.

3. Assay of triterpenoids

The dried mycelia (1 g) were suspended in 40 ml of ethanol (95%) and the extraction was assisted by successive sonication using Delta D200H Sonication Cleaner. After sonication at 50 °C for 1 h, the mycelia were removed by centrifugation at 6000 rpm for 15 min, the supernatant was filtered though a 0.45 μm membrane, the filtrate was analyzed for triterpenoids by HPLC. The HPLC system was composed of a Hitachi L-6200 solvent delivery controller, a Hitachi 4250 UV–vis detector, a Hitachi-D-2500 Chromato-integrator, and a reverse phase ODS column (ACE, UK; 250 mm × 4.6 mm, 20 μm). The injection volume was 20 μl, and the gradient elution was performed using acetonitrile (A) and 0.1% phosphoric acid in water (B). The mobile-phase composition was changed linearly from 0% A to 90% A in 90 min. The flow rate was set at 1 ml/min, and the eluent was monitored at 254 nm [18,19]. Ginsenoside Rc (Sigma, St. Louis, MO) was used to construct a calibration curve from which triterpenoid concentrations were determined.

4. Results and discussion

4.1. Effect of nitrogen sources on cell growth and the production of metabolites

Effects of different nitrogen sources, i.e. malt extract, yeast extract and corn steep powder, on the cell growth and the production of components such as EPS, IPS and triterpenoids by *A. cinnamomea* CCRC36716 were investigated. As shown in Table 1, the three nitrogen sources (ME, YE and CSP)