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The cultivation strategy of enhancing triterpenoid production in submerged cultures of *Antrodia cinnamomea* by adding monoterpenes

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ABSTRACT

Antrodia cinnamomea has long been used as a folk remedy; recently, it has become a very expensive medicinal mushroom in Taiwan. Triterpenoids are considered to be among the most biologically active components found in *A. cinnamomea*. The aim of this research was to investigate the feasibility of enhancing triterpenoid production in shake flask cultures of *A. cinnamomea* by adding various types of monoterpenes. The results demonstrated that a monoterpene ethanol solution was inhibitory in regard to mycelia growth; an appropriate time for their addition was determined to be on day 7. Of the various monoterpenes tested, limonene proved to be the most effective in enhancing polyphenol and triterpenoid production. A 1% (v/v) addition of limonene ethanol solution was found to be the most appropriate level; at this level, the content of crude triterpenoid rose to the highest level of 33.93 mg/g DW, for a three-fold increase. In contrast, total polyphenol content achieved the highest concentration of 11.02 mg/g DW at an adding level of 2% (v/v) limonene and 1% (v/v) ethanol, representing a nearly two-fold increase compared with the control group. Moreover, this study also demonstrates that monoterpene added into the media had been absorbed into the mycelia of *A. cinnamomea*, and triterpenoids could be one of the metabolic derivatives.

1. Introduction

The fungus *Antrodia cinnamomea*, is an exclusive parasitic fungus on the inner wall of the endemic species *C. kanehirai* Hay. It has been used as a folk remedy for food, alcohol and drug intoxication, as well as diarrhea, abdominal pain, hypertension, skin itching and liver cancer [1,2]. Many researchers have revealed that *A. cinnamomea* possesses antioxidant, antitumor and immunomodulating activities [3–7]. Over 78 compounds, including: terpenoids, benzenoids, lignans, benzoquinone derivatives, succinic and maleic derivatives have been identified, and many of these compounds were evaluated for biological activity [8]. Among these compounds, triterpenoids have recently been considered as the most biologically active components and potential anticancer agents due to their activity against tumor growth [8]. Owing to such physiological functions, determining how to increase the production of triterpenoids by controlling the cultivation conditions or modifying the media compositions deserves further study in detail.

Because wild fruiting bodies of *A. cinnamomea* grow extremely slowly, it has become a very expensive medicinal mushroom in Taiwan. As a result, submerged cultivation has developed as an alternative to commercial practice at the present time. The use of submerged cultures provides a number of potential advantages, including higher mycelial production in a more-compact space over a shorter time with a lower chance of contamination, and as the preferred route for the production of a number of valuable metabolites. In order to enhance production efficiency, the modification of media compositions or the control of environmental conditions would be vital factors. The addition of bark extract or wood chips of *C. kanehirai* Hay has been demonstrated to be favorable to the growth of *A. cinnamomea* in mycelial cultures [9–12]. However, from a plant conservation point of view, finding a substitute for wood chips is a necessity. The hot water extract of the stem (*Cinnamomum camphora*) showed the strongest promotion of the mycelial growth. The petroleum ether extract showed the greatest stimulatory effect on content and production of triterpenoids. This study demonstrates that α-terpineol can act as an elicitor for triterpenoid biosynthesis in *A. Cinnamomea* [13]. Among many sources, citrus peels are the most familiar and a rich source of essential oils. According to our published papers, we proved that peel extract additive could effectively enhance the production of biomass and bioactive metabolites [14,15]. Some citrus peels deserve to be considered in regard to accelerating the production process, although the deal type and level have to be determined with caution. Their function could mainly be explained in terms of

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the chemical compounds in essential oils. The essential oils of these fruits are composed of a complex mixture of 200 natural plant components known as terpenes and terpenoids. For instance, mandarin peel oil is mainly composed of limonene (73%), γ-terpinene (17%) and β-pinene (1.5%) [16]. Therefore, some pure compounds categorized under monoterpenes will be chosen as additives in order to more fully understand their efficacy in this study.

2. Materials and methods

2.1. Organism and inoculum

A. cinnamomea CCRC35396 was obtained from the Bioresources Collection and Research Center (BCRC) at the Food Industry Research and Development Institute (Hsinchu, Taiwan). The strain was maintained on potato dextrose agar (PDA) slants. The slants were incubated at 25 °C for 7 days and then stored at 4 °C. The strain was transferred to a fresh PDA plate every month.

2.2. Inoculum preparation

The medium for the seed culture was made up of the following components (w/v): glucose 2.0%, malt extract 2.0% and peptone 0.1%. The pH value was initially adjusted to 5.5 with the addition of either 1 N NaOH or 1 N HCl, followed by autoclaving at 15 psi, 121 °C for 15 min. *A. cinnamomea* was transferred to the medium by punching out 0.7 mm diameter agar discs from the 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.

2.3. Shake flask cultures

The flask culture experiments were performed using 250 mL flasks containing 100 mL of basal medium inoculated with 10% (v/v) of the seed culture. The basal medium consisted of corn starch 4.78% (w/v) and YM Broth 3.19% (w/v) [17]. The initial pH value was adjusted to 5.5 with the addition of either 1 N NaOH or 1 N HCl. Apart from the basal medium, various kinds or concentrations of monoterpene ethanol solution were added to the medium to study their influence on the formation of bioactive metabolites. All media were sterilized at 121 °C for 20 min. The culture was incubated at 25 °C on a rotary shaker incubator at 100 rpm for 28 days; samples were collected at various intervals from the shake flasks for analyses of biomass dry weight, intracellular polysaccharides (IPS) and triterpenoid production. Due to the fact that pellets were formed from the submerged cultures of mycelium, taking a sample from a flask by a pipette was difficult or even impossible. Therefore, one flask was required for each assay and a fermented broth of 100 mL was used for the determination of mycelial concentration. Three sets of shake flasks were prepared at the same time for each test. The resultant values are the means of the triplicate determinations.

2.4. Preparation of monoterpene ethanol solution

According to the previous paper, citrus peel oil is mainly composed of limonene, γ-terpinene and β-pinene [16], which were chosen to be the additives in the tests carried out in this study. Since monoterpene is insoluble in the media, in order to be an additive, three kinds of monoterpene ethanol solution had to be prepared first by mixing monoterpene and ethanol with a ratio of one to one (V/V).

2.5. Determination of biomass and starch concentration

To determine the biomass concentration, the mycelia from a sample were filtered through a 30 μm pore-size mesh and washed with a large amount of distilled water, then collected by filtration through a pre-weighed Whatman filter paper no. 2 (Whatman International Ltd., Maidstone, UK), followed by freeze-drying to a constant dry weight. The values are the means of the triplicate determinations. A quantitative starch-iodine method was adopted for off-line determination of the starch concentration [18].

2.6. Measurements of intracellular polysaccharides

Intracellular polysaccharides (IPS) were extracted from the dried mycelia (100 mg) by suspending the mycelia in 10 mL of distilled water and autoclaving at 15 psi, 121 °C for 15 min [19]. The water extracts from the shake flasks were centrifuged at 8000 rpm for 10 min, and the resulting supernatant was mixed with four volumes of 95% (v/v) ethanol, stirred vigorously and left overnight at 4 °C. The precipitated polysaccharide was collected by centrifugation at 8000 rpm for 10 min, and then lyophilized to remove residual ethanol. Total polysaccharides in the culture medium were determined by a phenol–sulfuric acid assay according to Dubois et al. [20]. Owing to the interference from the presence of starch in the medium, extracellular polysaccharide was not assayed in this study.

2.7. Assay of crude triterpenoid

The determination of the crude triterpenoid content was similar to that described by Tsujikura et al. [21]. The dried mycelia (100 mg) were extracted by 50% (v/v) ethanol (3 mL) for 1 week (twice). After removal of the mycelia by centrifugation, the supernatants were dried at 50 °C under vacuum conditions. The residues were suspended in 3 mL water; 3 mL chloroform was then added for extraction. After removal of the upper water layer, the crude triterpenoid in chloroform was extracted by 5% (w/v) $NaHCO_3$. 2 M HCl was added to adjust the pH of the $NaHCO_3$ layer to below 3; the crude triterpenoid in the $NaHCO_3$ layer was then extracted with chloroform. After removal of the chloroform by evaporation at 40 °C, the crude triterpenoid was dissolved in absolute ethanol; its absorbance was detected at 245 nm in a spectrophotometer (Genesys UV10, Thermo, USA). The content of the crude triterpenoid was calculated on the basis of a standard curve prepared by using ursolic acid.

2.8. Gas chromatographic (GC) analysis

Essential oil components in the peel extract and monoterpenes were analyzed by gas chromatograph (Thermo model Focus GC series). The GC was equipped with a fused silica capillary column SGE BP20 (25 m x 0.22 mm i.d. x 0.25 μm thickness,), a flame injector detector (FID) with an operating temperature of 250 °C and an injector with a temperature of 250 °C [22]. The GC was operated under temperature programmed conditions from 50 °C (10 min) to 200 °C (10 min) at 1.5 °C/min. The gas carrier was nitrogen with flow rate of 25 mL/min, and the injection volume of sample was 1 μL.

2.9. Assay of total polyphenol

Total polyphenol content was determined according to the method of Singleton and Rossi [23] with a slight modification. The dried mycelia (100 mg) were extracted by methanol (20 mL) at 130 rpm for 12 h at 50 °C. Samples from the shake flasks were centrifuged at 8000 rpm for 5 min. The resulting supernatant (0.4 mL) was mixed with distilled water (9.6 mL) and Folin–Ciocalteu reagent (1 mL), and incubated for 5 min at 22 °C. After the addition of 5 mL of 5% sodium carbonate, the mixture was kept at 22 °C for 60 min, and its absorbance at 750 nm was measured against a blank. Determination of the content of total phenolic compounds was based on a standard curve of gallic acid.