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Reuse of citrus peel to enhance the formation of bioactive metabolite-triterpenoid in solid-state fermentation of *A. cinnamomea*

Fan-Chiang Yang[a,*], Te-Wei Ma[a,b], Ya-Han Lee[a]

[a] Department of Chemical and Materials Engineering, Tunghai University, 181, Section 3, Taichung Port Road, Taichung 40704, Taiwan
[b] Department of Chemical Engineering, Army Academy, 750 Longdong Road, Zhongli City, Taoyuan 32092, Taiwan

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ABSTRACT

Antrodia cinnamomea has recently become popular as a drug remedy in Taiwan because of the mushroom's physiologically beneficial properties. However, because of its host specificity, slow growth rate and rarity in nature, the fruiting bodies of *A. cinnamomea* have become the most *expensive* mushrooms in Taiwan in recent years. Artificial cultivation of *A. cinnamomea* basidiomes to satisfy market demand is considered to be the most effective solution.

In this study, solid-state fermentation was carried out in petri dishes for mycelia growth and basidiomatal formation of *A. cinnamomea*. Different kinds of citrus peel were added to the medium to investigate the feasibility of enhancing the production of bioactive compounds. The crude triterpenoid content of mycelia in the control test reached the highest level of 9.66 mg/g DW on day 30. Among various kinds of citrus peel, grapefruit peel was the most effective in enhancing the content of crude triterpenoid. Moreover, at the addition ratio of 4 g per petri dish, the amount of crude triterpenoid reached 47.10 mg/g DW on day 30, with more than a fourfold increase. Furthermore, compared with the mycelia of the control culture, the profiles of HPLC analysis show that the mycelia cultured with the grapefruit-peel addition contained more kinds of triterpenoid and was similar to natural basidiomes. This study demonstrates that additions of some kinds of citrus peel could effectively accelerate the formation of basidiomes and enhance the production of bioactive metabolites.

1. Introduction

Antrodia cinnamomea is an exclusive fungal parasite on the inner wall of the endemic species *Cinnamonum kanehirai* Hay. Fruiting bodies of *A. cinnamomea* are used as a traditional medicine in Taiwan. Because fungal basidiomes are so rare and attempts to cultivate them have failed, in recent years, *A. cinnamomea* has become an extremely expensive medicinal mushroom in Taiwan that is widely used in the treatment of various cancers and liver diseases. A number of reports have indicated that *A. cinnamomea* possesses antioxidant, antitumor and immunomodulating capacities. Many types of bioactive compounds identified in the fruiting bodies of *A. cinnamomea* include polysaccharides, sesquiterpene lactone, steroids and triterpenoids [1–4]. Apart from polysaccharides, triterpenoids were recently considered as one of the most biologically active components.

At present, small molecular bioactive components of *A. cinnamomea* research focuses on triterpenoids. In 1995, three new ergostane-type triterpenoids, Antcin A, Antcin B, and Antcin C were isolated from *A. cinnamomea*. Preliminary pharmacological studies revealed that Antcin A showed cytotoxicity against P388 murine leukemia and Antcin B exhibited anti-cholinergic and anti-serotonergic activities [1,2]. It has also been shown that triterpenoids extracted from *A. cinnamomea* have anticholinergic and anti-serotonergic activities. When four novel ergostane-type triterpenoids were isolated from the fruiting body of the fungus *A. cinnamomea* by Cherng et al. [4] and Dai et al. [5], it was reported that both mycelium and sporocarp of *A. cinnamomea* protect against acute liver damage induced by ethanol. Organic-solvent extracts from fruiting bodies of *A. camphorata* are rich in steroids and triterpenoids. The ethyl acetate extract of *A. cinnamomea* fruiting bodies possessed anticancer and anti-inflammatory activities [6,7] and the ethanol extract could induce HL 60 cells apoptosis [8]. The three ergostane-type triterpenes isolated from the fruiting bodies of *A. camphorata* displayed the most potent cytotoxic effect with an IC50 value of 22.3–75.0 μM. The compounds were also demonstrated to induce apoptosis in HT-29 and SW-480 cells, as confirmed by sub-G1 cell cycle arrest [9]. Owing to such physiological functions, determining how to increase the production of triterpenoids by the control of cultivating conditions or modification of media compositions deserves further study in detail.

* Corresponding author. Tel.: +886 4 23590262x214; fax: +886 4 23590009.
E-mail addresses: fcyang@thu.edu.tw, fcyang@mail.thu.edu.tw (F.-C. Yang).

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In the last decade, several researches have investigated the effect of cultural condition or medium compositions on the bioactive metabolites in the submerged cultures of *A. cinnamomea*. Most of related papers have been emphasized on how to enhance the production of polysaccharide by *A. cinnamomea* [10–13]. It has been reported that the production of triterpenoids of *A. cinnamomea* can be enhanced dramatically by means of modifying the medium's composition. Of all carbon sources tested, the highest overall triterpenoids production (31 mg/g DW) was obtained after 14 d of cultivation when 2% glucose was used [13]. According to response surface methodology (RSM), the results indicate that when a submerged culture in shake flasks was operated at 28 °C, initial pH 5.5, and rotation speed 105 rpm, the triterpenoid content in dry basis could be increased to 31.8 mg/g [14]. Two optimization models namely response surface methodology (RSM) and artificial neural network (ANN) were built to optimize the inoculum size and medium components for intracellular triterpenoid production from *A. camphorata*. The triterpenoid production experimentally obtained using the ANN–GA designed medium was 64.79 mg/l which was in agreement with the predicted value [15]. The results obtained are useful in regulation and optimization of *A. cinnamomea* culture for efficient production of cell mass and bioactive metabolites in the submerged culture.

In contrast, very little information is available concerning the influence of environmental conditions or medium compositions on the formation of biactive componets in solid-state fermentation of *A. cinnamomea* [16–19]. *In vitro* culture of *A. cinnamomea* on agar plates to induce fruiting body formation has been shown difficult since many of its physiological and developmental processes are unclear. The report has indicated that one dikaryotic isolate from a pairing between two compatible monokaryons obtained from two different basidiomes was able to produce basidiomes on PDA and MEA media at temperatures of 20–28 °C within 45 days of incubation. The basidia and basidiospores produced on the hymenial layer were microscopically observed [16]. Physical wounding of red hyphae was found to induce fruiting body formation on agar plate. Methanol extracts of white, red hyphae, wildly grown and *in vitro* grown fruiting bodies analyzed by HPLC showed a distinct pattern between hyphae and fruiting bodies [17]. It was reported that using 10 known components in *A. cinnamomea* including 5 ergostanes (Antcins C and K, and zhankuic acids A, B, and C), 4 lanostanes (sulfurenic acid, dehydrosulfurenic acid, eburicoic acid, and dehydroeburicoic acid), and 1 monophenyl (4,7-dimethoxy-5-methyl-1,3-benzodioxole) as standards, mycelia and basidiomes of *A. cinnamomea* were differentiated. Natural basidiomes and cultured basidiomes both contained all 10 test components. However, natural mycelia and cultured mycelia both contained the 4 lanostanes and 1 monophenyl but not the 5 ergostanes. These results indicate that the production of ergostanes is related to basidiomatal formation of *A. cinnamomea*, but is not related to the substrate on which the organism is grown [18]. However, very little information is available regarding solid-state fermentation for mycelia growth and basidiomatal formation of *A. cinnamomea* in petri dishes [16,18,19].

As described above, *A. cinnamomea* specifically grows on the trunk of a tree endemic to Taiwan, *Cinnamomum kanehirai* Hay. This native host species is becoming scarce, leading to difficulty in finding the fruiting bodies of *A. cinnamomea* in the field. It has also been shown that *Cinnamomum kanehirai* essential oil provides a favorable environment that promotes the growth of *A. cinnamomea* but inhibits other possible competitive fungi, for better colonization of the fungi on their individual hosts, but they are not essential for basidiomatal formation of the fungi [19]. From the plant-conservation point of view, finding a substitute replacement for the wood chips deserves further study. Among many sources, citrus fruit peels are the most familiar and a rich source of essential oils. This research focused on exploring the addition of different kinds of citrus peel to the medium as a means of enhancing the production of bioactive compounds of *A. cinnamomea* in petri dish solid-state cultures.

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.

2.2. Inoculum preparation

The medium for seed culture was made up of the following components (w/v): glucose 2.0%, malt extract 2.0% and peptone 0.1%. The pH was initially adjusted to 5, 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 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.

2.3. Solid-state fermentation in petri dish

Solid-state fermentation was performed in a petri dish. The basal medium used in this study was made up of buckwheat powder 28 g and distilled water 20 mL. Apart from the basal medium, various kinds or concentrations of citrus peel powder were added to the medium to study their influence on the formation of bioactive metabolites. After sterilization at 121 °C for 20 min and cooling, 10% inoculum was added to the petri dishes, which were subsequently incubated at 25 °C in the dark for around one month for mycelia growth and basidiomatal formation. Apart from the basal medium, various kinds and different concentrations of citrus peel powder were added to the medium to study their influence. At the end of incubation, the mycelial layer at the top of the culture was removed from the petri dish to analyze biomass dry weight, intracellular polysaccharides (IPS), total polyphenols and triterpenoid production. One petri dish was required for each assay, and three sets of petri dishes were prepared at the same time for each test. The values are the means of triplicate determinations. All test samples were completely dried in an oven at 50 °C before being extracted with ethanol.

2.4. Preparation of citrus peel powder

Four kinds of fruit (tangerine, lemon, orange and grapefruit) were purchased from a Taichung local market. Some 50 g of peel were dried at 60 °C to a constant weight and then ground into powder for medium additives.

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-weighted 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 triplicate determination. A starch-iodine method was adopted for off-line starch-concentration analysis [20].