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Polyhydroxylated steroids and triterpenoids from an entophytic fungus, *Hypocreales* sp. NCHU01 isolated from *Tuber magnatum*

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ABSTRACT

The submerged mushroom fermentation process has been used to obtain the mycelial biomass and metabolites for the production of pharmaceutical compounds. In this study, the approach was developed to produce mycelial biomass and triterpenoids in the submerged culture of the truffle entophytic fungus *Hypocreales* sp. NCHU01. The carbon and nitrogen sources were found to significantly influence the biomass and triterpenoid production. In the carbon source test, 10 g/L of sucrose yielded the highest triterpenoid production, reaching 132.26 mg/L. In the nitrogen source test, 15 g/L of yeast extract was identified as the most favorable source for crude triterpenoid production, reaching 157.68 mg/L. In the stimulatory effect, the addition of 2 g/L linoleic acid or 125 mg/L chitosan to the medium was found to elevate triterpenoid production to 327.5 mg/L and 343.2 mg/L, respectively. The major sterol compositions of the fruiting body of *Tuber magnatum* and mycelia of *Hypocreales* sp. NCHU01 were identified by gas chromatography with a mass spectrometer. It was found that sterol composition in the fermentation mycelia of *Hypocreales* sp. NCHU01 was quite similar to that of the fruiting bodies of *Tuber melanosporum*, *T. magnatum* and *T. sinense*. Two kinds of sterols (*i.e.* barrigenol R1; and dehydroepiandrosterone) were identified for the first time from Tuber species in this work. This work proposed the cultivation of entophytic fungus *Hypocreales* sp. NCHU01 as an alternative resource for *T. magnatum* from the viewpoint of sterol production.

1. Introduction

Truffles, belonging to the Tuber genus, are a hypogeous fungus that establishes an ectomycorrhizal symbiosis with trees and shrubs. The distinctive aroma of truffles is the main reason for their use as a delicacy in many famous cuisines, such as French, Italian and Spanish. Among the ascomycete truffles, *Tuber* and *Terfezia* are known as excellent edible fungi with considerable economic importance [1]. In addition to their nutritional importance, aroma and flavor, truffles represent a vast and yet largely unexploited source of therapeutic compounds with anti-inflammatory, antioxidant, antimicrobial, immune-suppressor, anti-mutagenic and anti-carcinogenic properties. In fact, truffles are also a rich source of protein, amino acids, fatty acids, minerals and carbohydrates [2].

Numerous volatiles, such as dimethyl sulfide, dimethyl disulfide, methional, 3-methyl-1-butanol, 1-hexen-3-one and 3-ethylphenol have been identified in various truffle species [3]. Report also mentioned that bis(methylthio) methane is the main contributors to the distinctive aroma of *Tuber magnatum Pico* [4]. All of these compounds are commonly used in food production processes to imitate prestigious truffle organoleptic properties [5,6]. The investigation on the nutritional profile of truffles has shown that fresh *T. aestivum* and *T. magnatum* are a rich source of proteins (11–12.9% and 20.5–24%, respectively), and carbohydrates (5.65% and 2.23%, respectively) [7]. In addition, reports also mentioned that the desert truffles *Terfezia* and *Tirmania* spp. could produce secondary metabolites with antiviral, antimicrobial, anti-mutagenic, antioxidant and anti-inflammatory activities [8,9]. Many steroids such as cholesterol, ergosterol [10], brassicasterol, lanosterol, campesterol, β-sitosterol, phytosterols [11] and other compounds such as fatty acids [12] and sphingolipids [13–15] were found to exist in the fruiting-body of *Tuber* species. Among them, the complex composition of the truffle steroids might be taken as the fingerprint for the identification of the truffle species.

Entophytes are microorganisms that live in the intercellular spaces of the tissues of plants or mushrooms without causing discernible manifestation of disease [16,17]. Recent statistical analyses showed that 51% of the biologically active metabolites obtained from entophytes were previously unknown, compared with

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only 38% of novel substances from soil microflora [18]. Entophytes have been regarded as a prolific source of structurally novel secondary metabolites with good biological activities [19,20], *e.g.*, the production of taxol, an anticancer diterpene first obtained from *Taxus brevifolia*, by fungi in an artificial culture medium [21]. In the literature, truffles are known as complex microhabitats hosting bacteria [22], yeasts [23] and filamentous fungi [24] in their gleba. Pacioni et al. reported that several truffles, such as *T. rufum*, *T. brumale*, *T. magnatum*, *T. melanosporum*, *T. nitidum* and *T. excavatum* were found to host abounding fungi. Characterization of the truffle guest fungi according to morphological and molecular analyses revealed that the entophytic fungi were mostly belonged to the Eurotiales, Hypocreales, Basidiomycota, Helotiales and Mucorales. Three kinds of entophytic fungus: *Paecilomyces fumosoroseus*, *Verticillium insectorum* and *Verticillium leptobactrum*, belonging to the Hypocreales order have been isolated from *T. magnatum* [24]. In our lab, an entophytic fungal strain isolated from the fruiting body of *T. magnatum Pico* was identified as *Hypocreales* sp. according to its internal transcribed spacer (ITS) sequence. Based on the rDNA-ITS sequence analysis, together with morphologic traits, this strain was preliminarily grouped into the species of Hypocreales and named as *Hypocreales* sp. NCHU01. Meanwhile, the polysaccharides production of this strain had been revealed [25].

There were a few studies concerning about the chemical constituents, such as phenolics or sterols, produced by the entophytic strain [11,26]. Wang et al. showed that the cultures of *Colletotrichum* sp., an entophytic strain in *Ginkgo biloba*, gave new biologically active flavones with potent anticancer and antioxidant activities [27]. Among entophytic strains, only a few grass species have been studied for their activities and metabolites [28]. The use of these entophytic strains to produce special metabolites originally existing in the host is becoming more attractive due to the convenience and cost consideration in production.

Due to the slow-growing characteristics of the truffle, the feasibility of production of truffle metabolites such as steroids was the first time carried out with a truffle entophytic strain *Hypocreales* sp. NCHU01. This study tries to compare the presence of triterpenoid and steroid contents in truffle and its entophytic fungus. The production conditions were examined. The effect of the stimulators was tested to enhance metabolites production. GC–MS pattern was used as a fingerprint to identify steroid content. The similarity of steroid content between the truffle fruiting bodies and the mycelial biomass of this entophytic fungus was also conducted.

2. Materials and methods

2.1. Chemicals and reagents

All the solvents and chemicals used were of analytical grade. Cyclohexane, absolute ethanol and potassium hydroxide were purchased from Echo chemical Co., Ltd. (Taichung, Taiwan). N,O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA), cholorotrimethylsilane (TMCS) and anhydrous pyridine were purchased from Uni-Onward Corp. (Taichung, Taiwan). The internal standard, 5α-androstan-3β-ol was purchased from Sigma-Aldrich China Inc. (Taipei, Taiwan). The following authentic standards were purchased from J&K Scientific Ltd. (Beijing, China): cholesterol (95%), ergosterol (98%) and lanosterol (55%). The phytosterol mixture, *i.e.* brassicasterol (13%), campesterol (26%), stigmasterol (7%) and b-sitosterol (53%), was purchased from Zhongyitai Trade Co., Ltd. (Yichang, China). The saponification reagent, saturated aqueous KOH, was freshly prepared before use.

2.2. Microorganisms

The strain used in this study was isolated from the fruiting body of *T. magnatum Pico,* and was identified as *Hypocreales* sp. NCHU01 according to its internal transcribed spacer (ITS) sequence based on the rDNA-ITS sequence (GenBank accession number HQ608125). It was maintained on a potato-dextrose-agar slant. The slant was inoculated with mycelia and incubated at 25 °C for 30 days. The seed media consisted of the following components: 0.5% glucose, 0.1% yeast extract, 0.1% peptone, 0.02% KH_2PO_4, 0.02% $MgSO_4{\cdot}7H_2O$ [29]. The cultivations were then conducted in 500 ml Erlenmeyer flasks containing 100 ml of seed media, and inoculated with two units of cutter square (20 mm × 20 mm), prepared by a self-designed cutter on a rotary shaker set to 100 rpm, 25 °C for 14 days.

2.3. Flask cultivation

A shake-flask culture was performed in a 250 ml Erlenmeyer flask containing 100 ml of the main medium. The main medium consisted of the following components (g/L): sucrose, 10; yeast extract, 15; KH_2PO_4, 0.5; $MgSO_4{\cdot}7H_2O$, 0.5; vitamin B1 hydrochloride, 0.15. A volume of 90 mL medium in a 250 mL shake flask was inoculated with 10 mL inoculums (with *ca.* 350–450 mg cell dry weight per liter, mg CDW/L). The pH was adjusted to the desired value by the addition of either 0.1 N HCl or 1 N NaOH. Media were sterilized at 121 °C for 20 min and the carbon source was autoclaved separately. The flasks were incubated on a rotary shaker at 100 rpm and 25 °C for 14 days.

2.4. Sample preparation

According to a previously described method [30], a Tuber powder sample (100 mg) mixed with 100 μl of internal standard (2 mg/ml of 5α-androstan-3β-ol ethanolic solution) was saponified with 0.5 ml of saturated aqueous KOH in 8 ml of absolute ethanol at 85 °C for 30 min. The unsaponifiable matter was extracted with 20 ml of cyclohexane from the hydrolysate diluted with 12 ml of water. One tenth of the extract was dried under a gentle nitrogen stream at ambient temperature, and the sterols were then derivatized to trimethylsilyl (TMS) ethers according to an optimized method [31] with minor modifications. In brief, 125 μl of BSTFA (with 1% TMCS) and 125 μl of anhydrous pyridine were added to the dry residue, and the mixture was shaken for approximately 90 s and heated at 70 °C for 25 min. After cooling, 1 μl aliquots were directly injected into the gas chromatograph. All assays were performed in triplicate for each Tuber sample.

2.5. Assays

2.5.1. Cell biomass

Three flasks were taken each time. Dry cell weight was obtained by filtering the broth through a 30 mm pore size membrane. The cells were then washed three times with distilled water and dried at 60 °C to a constant weight.

2.5.2. Total triterpenoid analysis

A 5% vanillin-glacial acetic acid solution was freshly prepared before use. 0.5 g vanillin was transferred to a 5 mL volumetric flask, and a small quantity of glacial acetic acid was added. The solution was shaken until the vanillin was totally dissolved, and then diluted with glacial acetic acid to a volume of 10 mL. The solution was shaken until it was well-distributed and used freshly.

0.2 mL of the solution was added in the 10 mL tubes. Then, the solvent in each tube was evaporated at 70 °C in a water-bath. Following this, 0.2 mL 5% vanillin-glacial acetic acid solution and