Production of bioactive metabolites with pharmaceutical and nutraceutical interest by submerged fermentation of \textit{Pleurotus ostreatus} in a batch stirred tank bioreactor

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Abstract

Mushrooms comprise a vast source of new pharmaceutical and nutraceutical products. Submerged fermentation of the mycelial form of mushroom-producing fungi is promising for efficient production of their biomass and active metabolites. The aim of this study was the isolation and identification of bioactive metabolites derived from the edible mushroom \textit{Pleurotus ostreatus} biomass, produced by submerged fermentation in a batch stirred tank bioreactor. The dichloromethane extract was fractioned by medium pressure liquid chromatography (MPLC) and Sephadex LH-20 column chromatography. For extracting efficiently the phenolic compounds of the methanolic extract, an adsorption-desorption process, using XAD-4 type resin, was performed. The pure compounds were elucidated with 1D/2D NMR-spectroscopic analyses, NMR data comparisons, and chemical correlations combined with GC/MS-LC/MS experiments. The compounds afforded by the dichloromethane extract were identified as linoleic acid (1), oleic acid (2), stearic acid (3), palmitic acid (4) and their corresponding methyl esters (5-8, respectively), benzoic acid (9), trans 3, 4-dihydro-3, 4, 8-trihydroxynapthalen-1(2H)-one (10), 4-hydroxy-benzaldehyde (11), indolo-3-carboxylic acid (12) and uracil (13). The investigation of the methanolic extract led to the isolation of 3-formyl-pyrrole (14), 4-hydroxy-benzoic acid (15), uridine (16), nicotinic acid (17) and nicotinamide (18). Based on existing literature data, all these compounds exhibit valuable biological properties. The information obtained is considered fundamental for further investigation of the \textit{P. ostreatus} fermentation process on an industrial scale for enhanced bioactive metabolite production.

Keywords: Pleurotus ostreatus; submerged fermentation; stirred tank bioreactor; bioreactor metabolites; FCPC
1. Introduction

Both wild and cultivated mushrooms have been known for their nutritional and culinary values as well as viewed as tonics and used as medicines by humans for ages. In modern terms, they can be considered as functional foods, which can provide health benefits beyond the traditional nutrients they contain [1].

Nutritionally, mushrooms are low in energy and fat but high in protein, carbohydrate, and dietary fibre. Interestingly, mushrooms can be considered as a potential new source of dietary fibres, since fungal cell walls are rich in non-starch polysaccharides, of which β-glucans are the most interesting functional components [2]. Moreover, mushrooms contain a variety of minerals and trace elements such as potassium, and copper and vitamins such as riboflavin, niacin, and folates [3].

In addition to their nutritional value, certain mushrooms are abundant sources of a wide range of useful natural products. Indeed, various compounds including terpenoids, steroids, phenols, alkaloids and nucleotides, which have been isolated and identified from the fruit body, culture mycelium and culture broth of mushrooms are shown to have promising biological effects, preventing a range of diseases predominant in Western developed countries, such as hypertension, hypercholesterolemia, diabetes and cancer [4].

*Pleurotus* having various biotechnological applications in medicine, food and drug industry is viewed promising as medicinal mushroom, exhibiting a wide range of biological activities [5]. *Pleurotus ostreatus* (Jacq.:Fr.) P. Kumm., also known as the oyster mushroom is a Basidiomycetes belonging to the family Pleurotaceae (Agaricales, Agaricomycetes). Interest in this species has increased considerably in the last decade because of its gastronomic value and its nutraceutical properties [6]. The medicinally beneficial effects of *P. ostreatus*, such as their antioxidant activities, immunomodulatory effects, antitumor activities, antiviral, anti-inflammatory, antibiotic and cholesterol-lowering activities, have been investigated intensively [5].

Currently, commercial mushroom products are mostly derived from the fruiting bodies of field-cultivated mushrooms, which is a time-consuming and labor-intensive process. It usually takes several months to cultivate the fruit body of mushrooms and it is difficult to control the product quality during its soil cultivation. Submerged cultivation of edible and medicinal mushrooms, which has received increasing attention around the world, is viewed as a promising alternative for efficient production of biomass and valuable metabolites. Specifically, it offers potential advantages of faster production for both mycelia biomass and metabolites, in a shorter time period within reduced space and lesser chances for contamination [7].

Considering the great interest for mushrooms as a functional food and source for the development of drugs and nutraceuticals, the objective of this study was the chemical investigation of the *P. ostreatus* ATHUM 4438 commercial strain, of Greek origin. Unlike relevant existing researches, concerning the isolation of natural products from fruiting bodies of mushrooms, this study presents the isolation and identification of compounds derived from biomass by submerged fermentation of the studied strain in stirred tank bioreactor. The information obtained in this work is considered fundamental and useful for the further development of the studied higher fungi fermentation process on an industrial scale, for enhanced bioactive metabolites production.

2. Materials and Methods

2.1. Biological Material

The strain ATHUM 4438 of *P. ostreatus* which was used for this study was obtained from the ATHUM Culture Collection of Fungi of the National and Kapodistrian University of Athens. *P. ostreatus* ATHUM 4438 is a commercial strain isolated from fruit bodies originating from a small-scale mushroom
farm in Siatista (Macedonia, Greece). The stock cultures of both strains were maintained on a potato dextrose agar (PDA) slants. Slants were inoculated, incubated at 25°C for 7 days and then stored at 4°C.

2.2. Media and fermentation conditions in bioreactor 20-L

The microorganism was initially grown on PDA medium in a petri dish, and then transferred to the culture medium by punching out 5 mm of the agar plate culture with a sterilized self-designed cutter. The composition of culture medium and fermentation conditions in bioreactor used, was the suggested for maximum biomass production, reported in our previous study [8]. The mycelial biomass was harvested, centrifuged, freeze-dried and powdered to ~1 mm particle size before analysis.

2.3. Extraction

Biomass powder of \textit{P. ostreatus} ATHUM 4438 was extracted using the method of Accelerated Solvent Extraction (ASE) performed on an ASE 300 system (Dionex, USA). The extraction was conducted consecutively with cyclo-hexane (c-HEX), dichloro methane (DCM), methanol (MeOH) and deionized water (DWR). The used extraction parameters were based on instrumental settings.

2.4. Isolation and Identification of compounds

The crude DCM extract (1 g) was subjected to medium pressure column chromatography (MPLC) carried out with a Sepacore MPLC system (Büchi C-650 pump) on normal phase silica gel (Merck 0.02-0.04mm) with a maximum applied pressure of 10 bar. The elution was conducted with a step gradient CH$_2$Cl$_2$/MeOH (100:0, 95:5, 90:10, 80:20, 50:50). MPLC analysis yielded a total of combined fractions: POMA1 (9.4 mg), POMA2 (231 mg), POMA3 (66.9 mg), POMA4 (47.5 mg), POMA5 (100.7 mg), POMA6 (16.8), POMA7 (176.9 mg), POMA8 (425.2 mg) and POMA9 (249.4 mg).

The chemical composition of non-polar fractions POMA1-POMA3 was analyzed using gas chromatography-mass spectrometric (GC-MS) technique. The mass spectrometer employed for GC-MS analysis was an HP 5973 mass selective detector in the electron impact (EI) ionization mode (70 eV); Hewlett-Packard 6890 gas chromatography; capillary column HP-5 MS (30m x 0.25mm; film thickness: 0.25μm capillary column, coated with phenyl-methyl siloxane) and HP Innowax (30m x 0.25mm; film thickness 0.50μm). The initial temperature of the column was 60 °C and then was heated up to 280 °C with a 3 °C/min rate. Injector and transfer line temperatures were set at 220 °C and 280 °C, respectively. Helium was used as carrier gas at flow rate of 0.6 mL/min. Each peak that was detected in the GC/MS analysis of POMA1-POMA3 fractions was searched and identified using the WILEY 275 mass spectral database, resulting in the identification of compounds 1-8.

The POMA5-POMA6 fractions were each separately submitted to Sephadex LH-20 (Aldrich) column chromatography and eluted with MeOH (100%) yielding compounds 9 (6.7 mg), 10 (4.93 mg), 11 (4.36 mg) and 12 (4.5 mg). Finally, fraction POMA7 was chromatographed on normal phase silica gel in medium pressure and eluted with a step gradient of CH$_2$Cl$_2$/MeOH (100:0, 95:5, 90:10, 80:20, 50:50). The MPLC analysis afforded compound 13 (7.9mg).

The MeOH extract (45.4 mg) was subjected to adsorption chromatography using as sorbent resin XAD-4 (purchased from Rohm and Haas) [9]. The phenolic (POXM) fraction (5.8 mg) obtained from this procedure was submitted to Fast Centrifugal Partition Chromatography (FCPC) which was performed on a Kromat FCPC unit with 1 L column capacity. The two-phase solvent system selected for the initial fractionation of the above fraction consisted of EtOAc:BuOH:EtOH:H$_2$O (3:10:5:15). The stationery phase consisted of the lower phase (polar) whereas the mobile phase was the upper (non-polar). For the preparation of the two-phase solvent system, each solvent was added to a separatory funnel and equilibrated at room temperature. The two phases were separated and the sample to be injected into the
column was dissolved in 50 mL of the lower phase. FCPC analysis yielded a total of combined fractions: A (77 mg), B (641.6 mg), C (304 mg), D (187.9 mg), E (313.6 mg), F (459.5 mg), G (789.2 mg), H (775.7 mg), I (397 mg) during ascending mode and fractions G (357.4 mg), K (395.9 mg), L (217.7 mg) and M (590.7 mg), during descending mode.

Further chromatographic separation of fraction C by Sephadex LH-20 column chromatography in which elution was carried out with MeOH (100%) resulted in 7 combined (POFA1-POFA7) fractions. Fraction POFA4 (26.8 mg) was subjected to preparative thin-layer chromatography (TLC) (ACN / CH2Cl2 / MeOH 75:5:25) to yield compound 14 (5.2 mg) and 15 (6.8 mg). Moreover, fraction POFA5 (10.3 mg) and POFA6 (12.2 mg) were purified by means of (preparative) HPLC. For this reason, it was used a Thermo Finnigan HPLC system (ThermoFinnigan, San Jose, USA), connected to a SpectralSystem UV2000 PDA detector with the incorporation of a Supelco A23, Discovery HS-C18 column (250 mm x 4.6 mm, 5 μm), while ChromQuest 2.1 software was used for the management of the data. The mobile phase composed of H2O–ACN (98:2 → 2:98) was gradient at a flow-rate of 1.2 mL/min and injection volume was 10μL. The effluent was monitored at 254 nm and peak fraction was collected according to the elution profile. The above analysis afforded compound 16 (3 mg) and 13 (4.5 mg). Furthermore, fraction D derived from FCPC analysis, was subjected to Sephadex LH-20 column chromatography, eluted with MeOH (100%) to obtain 4 fractions (POFB1-POFB4). Further chromatographic separation of fraction POFB3 (31.6 mg) by means of preparative TLC (ACN / CH2Cl2 / MeOH 75:5:25), led to the isolation of compounds 17 (14.7 mg) and 18 (11.5 mg).

Generally, all fractions were analyzed (examined) by TLC. Precoated TLC silica 60 F254 plates (Merck) were used (0.25 and 2 mm layer thickness for analytical and preparative TLC, respectively). Spots were visualized using UV light, and vanillin-sulphuric acid reagent. All compounds were identified by means of spectral data (1H-NMR and 2D NMR), HRMS and direct comparison with the respective literature data. 1H NMR (600 MHz) data were recorded on a Bruker Avance III 600 spectrometer CDCl3 and MeOD (Aldrich) as solvent and TMS as an internal standard. The 2D-NMR experiments (HMQC and HMBC) were performed using standard Bruker microprograms. Mass spectrometry APCI-HRMS were run on a LC/MS Thermo Scientific LTQ Orbitrap Discovery mass spectrometer.

3. Results and Discussion

3.1. Investigation of the DCM extract

The initial fractionation of the crude DCM extract of biomass derived from P. ostreatus growth in submerged culture in bioreactor was performed by means of MPLC analysis. The investigation of the resulting non-polar fractions by GC/MS and LC/MS analysis afforded the identification of 4 fatty acids and their derivatives. Specifically, the above investigation afforded linoleic acid (1), oleic acid (2), stearic acid (3), palmitic acid (4) and their corresponding methyl esters (5, 6, 7 and 8, respectively).

Further chromatographic analysis of the rest combined fractions obtained by the DCM extract, using the methods of MPLC and Sephadex LH-20 column chromatography, led to the identification of benzoic acid [10] (9), trans 3, 4-dihydro-3, 4, 8-trihydroxynaphthalen-1(2H)-one [11], 10, 4-hydroxy-benzaldehyde [12] (11), indolo-3-carboxylic acid [13] (12) and uracil [14] (13).

Based on existing literature data, all the isolates obtained from the fractionation and investigation of the DCM extract are regarded as functional food ingredients and of great interest in pharmaceutical industry, exhibiting numerous health benefits. For example, benzoic acid which is a phenolic compound widely used in food industry as a weak acid food preservative, it is also used for the treatment of fungal skin diseases [15]. On the other hand, polyhydroxylated α-tetralones, in which trans 3, 4-dihydro-3, 4, 8-trihydroxynaphthalen-1(2H)-one belongs, are known as metabolites implicated in the branched pathway of fungal DHN-melanin biosynthesis [16]. Similar naphthalenone metabolites have been previously isolated
from many Ascomycetes grown in submerged cultures, exhibiting activities against the virus *Herpex simplex* [17]. Moreover, 4-hydroxy-benzaldehyde, a phenolic compound widely used as flavor and fragrance agent in food and perfumery industry, has many pharmaceutical properties. For example, it is used in the industrial production process of amoxicillin (hemisynthetic penicillin), cephalosporin antibiotics and the antivomit trimethobenzamide [18]. Considering indolo-3-carboxylic acid, it is an alkaloid and like other indole derivatives have attracted particular attention due to their various pharmacological activities. For example Wu et al. [19] reported that indolo-3-carboxylic acid demonstrated significant activity against HIV replication in lymphocyte cells.

Considering the presence of the isolates obtained in this work in members of Basidiomycetes, it is already known that the main fatty acids found in members of Basidiomycetes are the reported ones, with the nutritionally valuable unsaturated fatty acids (oleic and linoleic acid) predominating in total fatty acid content [20]. In addition, Beltran-Garcia et al. [21] extracted from the fruit body of *P. ostreatus* some volatile compounds including benzoic acid, which exerted as well antibacterial activities. Considering the trans 3, 4-dihydro-3, 4, 8-trihydroxynaphthalen-1(2H)-one, until now, only the natural (-)-trans-isomer has been isolated from six different microorganisms. In addition, the natural cis-isomer has been isolated from the mutagenic microorganism *Verticillium dahliae* [22]. Even though similar naphthalenone metabolites have been previously isolated from many Ascomycetes grown in submerged cultures [17], the presence of 3, 4-dihydro-3, 4, 8-trihydroxynaphthalen-1(2H)-one in *P. ostreatus*, is reported for the first time. Furthermore, 4-hydroxy-benzaldehyde has been previously detected in *P. ostreatus* species, including *P. ostreatus* [23]. Although, indole-3-carboxylic acid was formerly isolated from many Basidiomycetes, with the most recent report in the Ascomycetes *Gaeumannomyces amomi* [24], it has not detected again in *P. ostreatus*.

### 3.2. Investigation of the MeOH extract

The extraction of phenolic compounds of the MeOH extract was performed by adsorption-desorption processes using XAD type resins. The chemical structure of the resin material favored adsorption by weak interactions of molecules with moieties of high electron density, such as aromatic rings. In contrast, sugars or polar lipids couldn’t establish this kind of interaction and were eluted with water flow during the rinsing phase. The adsorbed phenolic compounds were recovered by elution with MeOH, giving an enriched extract (POXM). As it was observed, almost 14% of the methanolic extract consists of phenolic compounds.

The investigation of the phenolic extract (POXM), based on the effective fractionation by FCPC analysis, afforded 3-formyl-pyrrole [25] (14), 4-hydroxy-benzoic acid [26] (15), uridine [27] (16), nicotinic acid (17) and nicotinamide (18).

All isolated compounds have specific interest. For example, 3-formyl pyrrole is a member of pyrrole alkaloids exhibiting anticancer, antibacterial and anti-inflammatory activities [28]. In addition, 4-hydroxy-benzoic acid and its derivatives, primarily known as the basis for the preparation of parabens using in cosmetics and pharmaceutical industries, find important applications as dietary antioxidants, natural flavours and medicines [29]. Moreover, uridine and its derivatives are viewed promising candidates as therapeutic agents for a variety of diseases [30]. Finally nicotinic acid and nicotinamide, which are forms of vitamin B3 are considered as important food supplements, while they have been also considered as important start material for the preparation of other biological active compounds [31].

Nicotinic acid and nicotinamide have been previously identified in *Pleurotus* mushrooms [32, 33], while 4-hydroxy-benzoic acid constitutes a common phenolic secondary metabolite of higher fungi. Interestingly, a potential biosynthetic route for the production of 4-hydroxy-benzoic acid is via the bioconversion of L-phenylalanine, an aromatic amino acid present in the nitrogen source used for the biomass production in this study (corn steep liquor), and which utilized satisfactory by *P. ostreatus*. L-
phenylalanine can be deaminated to trans-cinnamic acid by a phenylalanine ammonia lyase. Trans-cinnamic acid can be subsequently hydroxylated to β-hydroxyphenylpropionic acid, which in turn can be converted via a β-oxidation step to benzoic acid and then to 4-hydroxy-benzoic acid by the action of lignin peroxidase (LiP), an enzyme present in Pleurotus ostreatus cultures [34]. However, to the best of our knowledge, even though 4-hydroxy-benzoic acid has been formerly reported in many Pleurotus mushrooms [23], its presence in P. ostreatus is reported for the first time in this work. Finally, 3-formyl pyrrole is a natural product that is reported also for the first time in P. ostreatus.

References


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