Bioactive Metabolites in Liquid State Cultures

Subjects: Environmental Sciences

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Humans have appreciated mushrooms for their edible and medicinal value since the Neolithic age. Increased interest in the pharmaceutical potential of mushrooms has led to numerous publications that explore more than 100 reported medicinal functions. These are functions such as antifungal, antibacterial, antiviral, antioxidant, anti-inflammatory, anti-tumor, antiviral, anti-diabetic, anti-thrombotic, anti-allergic, antidepressive, antihyperlipidemic, digestive, hypotensive, cytotoxic, hepatoprotective, neuroprotective, nephroprotective, osteoprotective, and immunomodulating activities, among others. This therapeutic action is due to a wide array of bioactive metabolites isolated from the fruiting body, mycelium, and culture broth of macrofungi. Both complex high-molecular-weight compounds such as polysaccharides, proteins, and lipids and low-molecular-weight compounds such as terpenoids, polyketides, and alkaloids have been isolated and are under study. In addition to their pharmaceutical potential, mushrooms are recognized as a rich source of nutraceuticals. Nutraceuticals are substances that have positive effects, proven by clinical testing, on normal physiological functions that maintain health in humans, thus preventing and treating diseases and enhancing longevity. There is a globally growing market for nutraceuticals sold as nutrients and supplements or in the form of enhanced “functional foods”. Natural bioactive compounds found in macrofungi are ideal for this market and are already being used for their properties.

mushrooms biotechnology agricultural side streams metabolites

1. Carbohydrates: Polysaccharides (EPS and IPS) and Oligosaccharides

Polysaccharides are the most abundant carbohydrates found in nature; they are crucial for life maintenance in a variety of organisms such as plants, animals, microorganisms, and seaweeds. Polysaccharides are long-chain biopolymers composed of more than 10 monosaccharide units connected by glycosidic bonds, whereas oligosaccharides contain 3 to 10 monosaccharides [1]. Polysaccharides exhibit a wide variety of structures composed of different monosaccharides, glycosidic bonds, and functional groups. The precise chemical structure of polysaccharides can be investigated with a combination of analytical methods such as FTIR, NMR < Raman spectroscopy, GC, GC-MS, and HPLC. Their most common components are pyranoses such as glycose, galactose, mannose, xylose, and arabinose [2].

The diversity of their constituent monosaccharide composition and structure is responsible for their different biological activities. There is a close relationship between the polysaccharide’s structure and biological activity [3]. The different chemical structures and chain conformations are also responsible for the different activities of polysaccharides [4]. They play an important role in functions such as cell recognition, growth and differentiation, metabolism, embryonic development, immune response, energy storage, structural support, and antigenicity [5].

Polysaccharides found in fungi are made up of glucans, which are made of D-glycose monomers, two glucose units linked by α-glycosidic or β-glycosidic bonds. They all contain a β-linked glycose backbone, but the pattern and degree of branching vary from species to species. The α or β linkages can form heteroglycans such as mannose, galactose, arabinose, fucose, and xylose. Polysaccharides can also bind to proteins, protein residues, and peptides to create polysaccharide-protein complexes or peptide complex groups [6]. Microbial polysaccharides are categorized into intra-cellular polysaccharides (IPS) and extra-cellular polysaccharides (EPS), depending on their location. Due to the polysaccharides not being encoded in the genome and their structural complexity, there is little information available regarding their biosynthesis [7]. There are data on polysaccharides produced from 651 species of fungi [8] to which there are multiple confirmed biological functions attributed, including antidiabetic, antioxidant, immunomodulatory, hepatoprotective [9], antitumor, antiviral, anti-inflammatory, renal protective, and radioprotective actions [10], regulation of glycolipid metabolism and gut microbiota, anti-nephritic and antiangiongenic activity, and improvement of memory impairment [3].

Examples of mushroom polysaccharides isolated and characterized are pleuran from *Pleurotus* species, lentinan from *L. edodes*, ganoderan from *G. lucidum*, agaritine from *Agaricus* sp., grifolans from *G. frondose*, and calocyban from *Calocybe indica* [11]. Polysaccharides are major structural cell wall components of mushrooms, but they can be produced exo-cellularly in large amounts by the mycelium of macrofungi in liquid/submerged cultivation. This is not always the case, because the conditions required for their exo-cellular production are still under study [12]. Nevertheless, liquid cultivation of macrofungi mycelia results in much faster production of polysaccharides in comparison to fruitbody production and is a promising tool for mass-scale industrial production [13]. It should be mentioned that optimum conditions for EPS production can vary greatly for fungi in the same genus [14] and even between strains of the same species [15]. Apart from species and strain, the most important growth conditions for optimal EPS production are: type of culture, oxygen supply, temperature, pH, carbon and nitrogen source, content and ratio, salt content, and cultivation length [16][17][18][19]. Dedousi et al. (2021) used molasses as a substrate in liquid cultures of *Morchella* sp. producing IPS (4.8 g/L) and EPS (3.94 g/L) [17]. Diamantopoulou et al. report that adding OMW to the liquid substrate of *G. resinaceum* resulted in increased quantities of IPS (5.2 g/L) [18]. Many studies seek to optimize EPS production in liquid cultures by deploying a multitude of carbon and nitrogen sources, different light intensities, and wavelengths [20]. Homogenization of the mycelium inoculant by sonoporation proved to be effective in increasing EPS production and reducing the required quantity of starter mycelium in submerged cultures of *G. lucidum* [21]. It is important to mention that the production of EPS by fungi in liquid cultures is negatively correlated with IPS, indicating that the amount synthesized significantly decreases after the 8th or 12th day of culture [22]. IPS in liquid cultures of *V. volvaceae* were also found to start decreasing after the 8th day and continued to decrease until the end of the culture. IPS were produced in greater quantities when the substrate C/N ratio was 40 [23].

Beta-glucans are the most common polysaccharides found in the cell walls of Ascomycota and Basidiomycota [24]. Beta-glucans are groups of polysaccharides or dietary fibers composed of D-glycose monomers, linked by β (1→3) and β (1→6) glycosidic bonds [25]. Beta-glucans are classified as dietary fibers and prebiotics because they cannot be hydrolyzed by the gastric enzymes of mammals and reach the large intestine undigested, where they are fermented by the gut microbiota with potential health benefits for the host [26]. Beta-glucans cannot be synthesized by the human body, which leads to their recognition by the immune system, inducing both adaptive and innate immune responses. Current research has established that beta-(1→3,1→6)-D-glucans are efficient immunostimulant agents that can trigger a variety of molecular interactions that are not yet clearly understood [27]. It has been established that β-glucans can play a role in the prevention of insulin resistance, dyslipidaemia, hypertension, and obesity [28]. A recent systematic literature review of 34 clinical trials performed using fungal β-glucans summarized the main physiological impacts on patients as follows: a strengthened immune defense that reduces the incidence and symptoms of respiratory infections, improvement of allergic symptoms, improvement of mood states, and improvement of overall wellbeing. No adverse effects related to the supplementation of β-glucans were noted [29]. The content of fungi in beta-glucans varies from 16% to 53% and is influenced by factors such as species, strain, cultivation conditions, and total dietary fiber content. Several studies have studied the in vitro antibacterial and antiviral activity of beta-glucans through the activation of the non-specific immune system [30]. Beta-(1,3–1,6) glucans also exhibit a radioprotective effect, which has been investigated in vivo with mice [31] and has also been successfully used as a radioprotectant to treat and prevent radiation and chemotherapy-related injuries [32]. *P. ostreatus* fruiting bodies cultivated in agricultural residues contained high quantities of β-glucans (28.28% to 48.42% dw) [33]. Another study reports that the use of olive mill wastes in the substrate of *G. lucidum* resulted in up to a 43% increase in the β-glucan content of the produced mushrooms [34].

An extensive review of the mechanisms by which polysaccharides help the treatment of type 2 diabetes enumerates multiple compounds isolated from macrofungi [35]. Namely, submerged cultivation of *Laetiporus sulphureus* and *G. lucidum* produced polysaccharides with antidiabetic action in rats. A glucan-rich polysaccharide isolated from *Pleurotus sajor-caju* alleviated hyperglycemia and hyperinsulinemia in diabetic mice. Multiple polysaccharides from *Coriolus versicolor* successfully suppressed hyperglycemia, and polysaccharides from *G. frondosa* enhanced glucose metabolism and increased the synthesis of intracellular glycogen [35]. Lentinan has also been found to help prevent diabetes by protecting pancreatic β-cells [36]. Submerged liquid cultivation of *Inonotus obliquus* produced two novel polysaccharide fractions with hypoglycemic activity [37]. Polysaccharides from *T. versicolor* have been shown to alleviate bone deterioration caused by diabetes mellitus, in addition to improving hyperglycemic control [38].

Fungal polysaccharides inhibit tumor cell proliferation, improve body immunity, cause tumor cell apoptosis with direct contact, and prevent the spread and migration of tumor cells in the body [39]. The antitumor action of polysaccharides is mediated through a thymus-dependent immune mechanism, which involves the activation of cytotoxic macrophages, monocytes, neutrophiles, natural killer cells, dendritic cells, and chemical messengers, which triggers the complementary and acute phase responses [40]. More specifically, lentinan from *L. edodes* and schizophyllan from *Schizophyllum commune* have a similar structure and show effective antitumor and immune potentiating activity through the cytokine production from immunocytes. Schizophyllan is being commercially used in vaccines and cancer therapy [41]. Krestin from *C. versicolor*, maitake D-fraction from *G. frondosa,* and ganoderan from *Ganoderma* spp. are all polysaccharides that have shown antitumor activity in clinical trials [6]. Many mushroom polysaccharides are being utilized as a co-treatment along with chemotherapy because of their ability to prevent oncogenesis and tumor metastasis [24].

2. Proteins and Peptides

Proteins are high-molecular-weight polymers made of amino acids linked together by peptide bonds and are essential for human health. Increased population and current dietary trends have resulted in increased demand for protein and meat alternatives, and it has been proposed that mushroom proteins can efficiently substitute meat. Fungal proteins contain all nine essential amino acids required by humans, in addition to carbohydrates, vitamins, and carotenes. Mycoprotein is considered a high-quality protein comparable to animal protein since it provides a high protein-to-energy ratio. At the same time, it contributes to minimizing animal slaughter, reducing the carbon footprint of food production, and can be produced with low total costs independently of climate and landscape limitations [42]. *Pleurotus* spp. are well known for efficiently recycling agro-industrial wastes and producing biomass with a protein content of up to 49% of their dry weight [43]. Submerged cultivation of *P. sajor-caju* in 5-L stirred tank bioreactor using 10 g/L glycose resulted in fast production of mycelium biomass (8.18 g/L) with a high content of protein (32%) [44]. Similarly, *Cordyceps militaris* mycelium produced in submerged cultivation had higher protein content than that of the fruiting body (21.10% vs. 18.47%), and higher total essential amino acid concentration than that found in eggs [45]. *A. bisporus* cultivated in shake flasks with synthetic media produced 12.67 g/L biomass containing 36% *w*/*w* protein and noteworthy amounts of IPS (2.57 g/L) in only 26 days [46]. The screening of Basidiomycetes: *A. aegerita*, *Pleurotus sapidus*, *L. edodes*, *Wolfiporia cocos*, *Stropharia rugosoannulata*, *P. sajor-caju*, and *P. salmoneostramineus* grown in submerged cultures using industrial side streams as the sole carbon successfully converted the residues into biomass containing protein enhanced with lipids, carbohydrates, and vitamin D_2. *P. sapidus* excelled in the screening and produced biomass containing 21% true protein, 4% lipids, and 115 µg/g vitamin D_2 in 4 days [47]. Making use of statistical optimization, *Morchella fluvialis* mycelium with 38% *w*/*w* protein content was produced in liquid culture, deeming it a promising source of essential amino acids for the human diet [48]. *Pleurotus* species cultivated on agro-industrial by-products produced a total of 22 free amino acids in quantities up to 130.12 mg/g dw plus the neurotransmitter GABA and ornithine [49]. The connection between dietary protein, the gut microbiota, and overall human health is also being investigated [50], and a single band protein (HEP3) isolated from *H. erinaceus* was found to improve the immune functions of mice by regulating the gut microbiota [51].

Apart from dietary proteins, macrofungi produce various bioactive proteins and peptides classified as lectins, fungal immunomodulatory proteins (FIP), ribosome-inactivating proteins (RIP), antimicrobial proteins, and ribonucleases. Lectins are nonimmune proteins or glycoproteins that bind specifically to carbohydrates and result in cell agglutination. This property of lectins makes them useful in studying the structure of cell surfaces and the isolation and purification of polymers with carbohydrate units [52]. In the last years, many lectins with antiproliferative, antitumor, cytotoxic, and immunomodulatory activities have been isolated from edible mushrooms such as *A. bisporus*, *F. velutipes*, *G. lucidum*, *G. frondose*, and *V. volvacea* [53]. FIPs are a novel family of bioactive proteins isolated from macrofungi that exhibit significant activity in suppressing the invasion and metastasis of tumor cells and show promise as immunologic adjuvants for tumor treatment [54]. RIPs are enzymatically active proteins that can inactivate ribosomes by eliminating adenosine residues from rRNA. They have been studied for their ability to inhibit HIV replication and viral disease prevention and are promising substances for developing drugs [55]. Considerable antifungal activity at the RNA level is attributed to ribonucleases isolated from mushrooms [56]. A ribonuclease isolated from *Tuber indicum* inhibited the proliferation of breast cancer cells [57], and another ribonuclease from *G. lucidum* suppressed autophagy and triggered apoptosis in colorectal cancer cells [58]. Amanitins are cyclic peptides of eight amino acids that are well known for their toxicity, but they have shown very promising results in anti-cancer pre-clinical trials [59].

3. Fatty Acids and Lipids

All fatty acids have important roles in the human body, and thus human diet must include foods containing fatty acids [60]. Macrofungi contain a wide variety of fatty acids, but the biggest percentage are polyunsaturated fatty acids (PUFA) and specifically linoleic acid, which is an important essential ω-6 fatty acid that cannot be synthesized in the body [61]. The PUFA contents of mushrooms are greater than those found in cattle and pork while containing low amounts of saturated fatty acids (SFA) [61]. PUFAs have been shown to reduce serum cholesterol [62] and play an important role in the regulation of cell physiology and cardiovascular health [63]. The fatty acids found in *P. ostreatus* were 28 in total, but linoleic acid was in the highest concentration (57–81%), followed by oleic (6–20%), and palmitic acid (8–12%) [15]. Agitated submerged cultures of *G. applanatum*, *M. esculenta*, *A. aegerita*, and *P. pulmonarius* produced significant quantities of unsaturated fatty acids [22]. Agitation of the shake flasks has been reported to have a positive impact on lipid biosynthesis by macrofungi [64]. *Tuber aestivum* cultivated in liquid cultures containing rice cereal hydrolysates as the sole carbon source produced the highest amount of lipids compared to *M. vulgaris*, *M. elata L. edodes*, and *A. bisporus* in substrates of agro-industrial waste [16]. *Volvariella volvacea* biomass produced in synthetic media contained 0.7 g/L of total lipids with linoleic acid being predominant. The researchers report that lipid synthesis was independent of the C/N ratio and that the considerable amounts of lipids (32% *w*/*w*) synthesized early in the culture started decreasing after the 8th day until they reached 3% by the end of cultivation. Fractionation of the produced lipids revealed that most of the lipids produced were neutral (65% *w*/*w*), followed by glycolipids and sphingolipids (30% *w*/*w*), and phospholipids in negligible amounts (2–3% *w*/*w*). The composition of fatty acids was not significantly influenced by the different C/N ratios, contrary to that of neutral lipids which increased in the high C/N ratio substrate [65].

The major sterol found in macrofungi is ergosterol, which is a well-known steroid precursor of vitamin D2 and a fundamental molecule for many biological processes. When commonly consumed mushrooms such as *P. ostreatus*, *A. bisporus*, and *L. edodes* are exposed to UV radiation for a short time (1–3 s), ergosterol is transformed into vitamin D2 in nutritionally relevant amounts [66]. Ergosterol has also been linked with preventive action against cardiovascular diseases and antioxidant properties[67]. Cultivation of macrofungi on agri-food residues produced fruiting bodies with enhanced quantities of ergosterol (11–26 mg/g) [33]. *P. citrinopileatus* cultivated on grape marc plus wheat straw produced significant quantities of ergosterol (5.53 mg/g dw) [68]. Other polyunsaturated fatty acids found in mushrooms, tocopherols, are natural antioxidants with high biological activity against degenerative malfunctions, and microbial and cardiovascular diseases [50]. Ceramides are waxy lipids composed of sphingosine, a fatty acid with application in foods and cosmetics. Novel ceramides have been isolated from *G. frondosa* and *Armillaria mellea* [8]. The lipids produced by macrofungi could also be utilized in the development of healthier and more sustainable food products [69]. It should be added that the fatty acids and their metabolic products are partially responsible for the characteristic umami aroma and flavor compounds of mushrooms [70].

4. Phenols

A wide variety of mycochemicals are grouped under phenolic compounds and contain the characteristic aromatic ring bearing one or more hydroxyl groups. Phenolic acids are the main phenolics found in macrofungi and specifically ferulic, sinapic, caffeic, and p-lo-coumaric acids. These phenolic acids are mostly bound in complex structures such as esters. [71]. Dedousi et al. performed detoxification of molasses using *Morchella* species in liquid agitated and static cultures producing impressive amounts of biomass (up to 18.16 g/L) with a maximum content of fungal phenolics of 32.2 mg/g. Although different ratios of C/N in the substrate were used, the total phenolics content of the produced biomass fluctuated without any apparent trends [17]. The biomass containing fungal phenolic compounds also exhibits antioxidant activity that is strongly correlated with the phenolics content. Specifically, a novel *G. resinaceum* strain was cultured in a liquid medium containing olive mill wastewater (OMW), and the resulting biomass contained a total phenolic content of 508.5 μg GAE/g dw [18]. A study comparing the total phenolics and their composition in the fruiting bodies versus the submerged culture mycelium of *Coprinus comatus* and *Coprinellus truncorum* reports that submerged culture mycelium contained the highest amount of total phenolics. The phenolic profiles of the fruiting bodies and the cultured mycelium of the species exhibited high variation and diversity with vanillic, gallic, gentisic, and cinnamic acids being the most abundant [72]. Phenolics are well-known antioxidants and additionally exhibit antiallergenic, antiatherogenic, anti-inflammatory, antimicrobial, antithrombotic, cardioprotective, and vasodilator effects [67]. Although hundreds of studies report various flavonoid contents in macrofungi, Gil-Ramirez et al. categorically declare that mushrooms do not contain flavonoids because they lack the enzymes required to synthesize flavonoids [73]. The antioxidant action of phenolic compounds and their ability to be produced by microorganisms in liquid cultures has gained the attention of researchers as an environmentally friendly alternative to the chemically synthesized antioxidant phenolics widely used in the food industry [74]. The liquid culture of higher fungi using agricultural residues as a substrate results in the production of high amounts of extracellular [75] and intracellular [76] phenolics, making this production method attractive especially for industrial applications.

5. Terpenes and Terpenoids

Ganoderma species are considered a panacea for all kinds of diseases in Chinese traditional medicine. They have been used to treat diseases of the liver, nephritis, hypertension, and hyperlipemia. Most of the known bioactive compounds found in *Ganoderma* are lanostane and ergostane pentacyclic triterpenes. Ganoderic acids especially have been studied extensively in vitro for their ability to induce cancer cell apoptosis [77]. In the past years, several new lanostane triterpenes have been isolated from *Ganoderma* species exhibiting hepatoprotective activities [78], antimalarial [79], and antifibrotic activity [80]. Another interesting group of metabolites is the sesquiterpenoids as tremulane sequiterpenoids isolated from *P. igniarius* have shown vascular-relaxing activities [81]. Nine alliacane sesquiterpenes isolated from the submerged culture of *Inonotus* sp. BCC 22,670 exhibited antibacterial activity against *Bacillus cereus* and antiviral activity against Herpes Simplex Virus type 1 [82]. Sesquiterpenes isolated from a mycelial culture of *F. velutipes* were also found to have antimicrobial activity [83]. Novel terpenoids, erinacines, and corallocines, isolated from mushrooms of the genus *Hericium*, have been shown to induce nerve growth factor [84] and brain-derived neurotrophic factor [85], which makes them promising substances for treating neurodegenerative disorders such as Alzheimer's disease [86]. Cyathane diterpenoids produced by *Hericium* spp. exhibited antibacterial and cytotoxic activities [87] and enhanced neurotrophin induction in cell-based bioassays [88].

Carotenoids or tetraterpenoids are colored pigments ranging from light yellow to orange to deep red and they are produced by plants and microorganisms. They are powerful antioxidants that can scavenge free radicals, and thus can be used in food, as dietary supplements, and as cosmetic additives. Carotenoids have been found in the fruiting bodies of *Cantharellus cibarius* and *Cordyceps militaris* [52]. The novel carotenoids isolated from *C. militaris* were named cordyxanthins and are highly water-soluble, unlike the traditional hydrophobic carotenoids, a property that can extend their applications [89].

6. Nutrients

Macrofungi are also a good source of vitamins which are essential for human metabolism, immunity, and digestion. They contain above-average contents of ascorbic acid and B-complex vitamins such as thiamine (vitamin B1), riboflavin (vitamin B2), niacin, pantothenic acid (vitamin B5), pyridoxine (vitamin B6), folic acid (vitamin B9), vitamin A, and vitamin E. The levels of cobalamine (vitamin B12) in mushrooms are comparable to those in beef and liver. They also contain ergocalciferol, tocopherol, ergosterol, biotin, ergotheionine, and the minerals potassium, calcium, selenium, copper, magnesium, and other trace elements [67][90][91][92]. Although the contribution of mushrooms in the proposed daily values of nutrients is small, consistent consumption of mushrooms contributes to a nutritious diet and is associated with a higher intake of many nutrients [91].

7. Other Compounds

The rapid spread of antibiotic-resistant bacteria worldwide has led researchers to turn to compounds isolated from macrofungi. *Clitopilus passeckerianus* produces a tricyclic diterpenoid called pleuromutilin, which is a naturally occurring antibiotic and has a unique and highly specific mode of action, which inhibits protein synthesis in bacteria [93]. Extracts from *A. blazei, H. erinaceus,* and *G. frondosa* with documented effects against different diseases and infections were recently reviewed and presented as potential efficient tools against the lung inflammation that occurs with COVID-19 infection [94]. Comprehensive papers about the entirety of bioactive metabolites produced by macrofungi have been published by De Silva et al. [95], Schüffler [96], and Chen and Liu [97].

The synthesis of the above-mentioned major and minor metabolites is achieved through solid and/or liquid-state fermentations of lignocellulosic waste and agricultural bioproducts by macrofungi with high dietary and/or medicinal properties, procedures that are presented in detail in the following section.

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