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## Medicinal Mushroom Biotechnology

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# Medicinal Mushroom Biotechnology

Marin Berovic <sup>α</sup>, Bojana Boh <sup>ο</sup> & Andrej Gregori <sup>ρ</sup>

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## I. INTRODUCTION

A total of more than 200 medicinal functions are thought to be produced by medicinal mushrooms (MM) and fungi including antitumor, immunomodulating, antioxidant, radical scavenging, cardiovascular, cholesterol-lowering, antiviral, antibacterial, anti-parasitic, antifungal, detoxification, hepatoprotective, anti-diabetic, anti-obesity, neuroprotective, neuroregenerative, and other effects. Also, substances derived from MM can be used as painkillers or analgetics [4, 5, 6]. The best implementation of MM drugs and dietary supplements has been in preventing immune disorders and maintaining a good quality of life, especially in immunodeficient and immuno-depressed patients, patients under chemotherapy or radiotherapy, patients with different types of cancers, chronic blood-borne viral infections of Hepatitis B, C, and D, different types of anemia, the human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS), herpes simplex virus (HSV), Epstein Bar virus, Influenza viruses A and B, H5N1 [7], COVID-19 [8-10], West Nile virus, chronic fatigue syndrome, patients with chronic gastritis and gastric ulcers caused by *Helicobacter pylori*, and people suffering from dementia (especially Alzheimer's disease) [1, 2, 3, 5].

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To combat these threats, humankind is focusing more and more attention on mushrooms and mushroom products. Mushrooms themselves are consumed regularly as part of the human diet and are treated as healthy or functional foods. On the other hand, the term mushroom nutraceuticals or dietary supplements has been applied to products derived from medicinal mushrooms that are taken to enhance general health and fitness but are not a regular food but a dietary food supplements [1].

## II. MAIN PHARMACEUTICALLY ACTIVE COMPOUNDS

The main components of these supplements are polysaccharides, triterpenes and immunomodulatory proteins. Polysaccharide components, in particular, have been widely investigated as a source of anti-tumor and immunostimulating agents. They are widely distributed in mushrooms, with over 660 species from 183 genera reported to contain pharmacologically active polysaccharides. About 77% of all medicinal mushroom products are derived from the fruiting bodies, which have been either commercially farmed or collected from the wild, 21% from culture mycelium and 2% from culture broths. Precisely how these products work is still a matter of conjecture, but numerous laboratory animal tests as well as human clinical trials have shown them to be effective. In some cases, attention has focused on a single bioactive mushroom component and its effectiveness in treating specific disease conditions, much like a pharmaceutical. In the case of nutraceuticals/dietary supplements, emphasis has been placed on a combination of components that collectively impact on an individual's overall health and quality of life [2].

Many such products are currently available, and their market value worldwide increased from 1.2 billion in 1991 to 3.6 billion USD in 1994. The combined market value of medicinal mushrooms, mushroom extracts and derived products in 1999 was estimated to be 6.0 billion USD. That year, the United States nutraceutical market alone was valued at 35 million USD. Since then, demand has increased between 20% and 40% annually depending on the species, with *Ganoderma*-based dietary supplements alone valued at 1.6 billion USD. The MM industry has grown from small-scale (cottage-based) operations aimed at supplementing household incomes, to medium and mega-sized industrial

ventures. This review examines the past, present and future of MM development, and includes a pyramid model addressing key issues [3].

They are of different chemical composition, such as polysaccharides, glycopeptide-protein complexes, proteoglycans, proteins and triterpenoids, with most scientific attention focussed to the group of non-cellulosic  $\beta$ -glucans with  $\beta$ -(1-3) linkages in the main chain of the glucan, and additional  $\beta$ -(1-6) branch points, that are characteristic for the antitumor and immuno-stimulating action. Mushroom polysaccharides do not attack cancer cells directly, but produce their antitumor effects by activating different immune responses in the host. Their mechanisms of action involve them being recognized by several immune cells receptors as non-self molecules, so the immune system is stimulated by their presence. Structurally different  $\beta$ -glucans have different affinities toward receptors and thus generate different host responses [12].

Immunomodulating and antitumor activities of these metabolites are related to immune cells such as hematopoietic stem cells, lymphocytes, macrophages, T cells, dendritic cells, and natural killer cells, involved in the innate and adaptive immunity, resulting in the

production of biologic response modifiers [13]. Clinical evidence for antitumor and other medicinal activities come primarily from some commercialised purified polysaccharides, such as lentinan from Shiitake - *Lentinula edodes*, krestin from *Coriolus versicolor*, grifolan from *Grifola frondosa*, and schizophyllan from *Schizophyllum commune* [12,14], but polysaccharide preparations of some other medicinal mushrooms also show promising results.

### III. WOOD DEGRADING *BASIDIOMYCETES* WITH PHARMACOLOGICAL EFFECTS

Almost unknown in Western Scientific Research only three decades ago, some of the wood degrading *Basidiomycetes* became intensely and systematically studied due to their promising pharmacological effects (Figures 1 A-E). Among them, *Ganoderma lucidum* – Ling zhi or Reishi, *Grifola frondosa* – Maitake, *Hericium erinaceus* – Lion mane and *Cordyceps militaris* have been known from the traditional Asian medicine, and *Trametes versicolor* Turkey tale, (previous *Coriolus versicolor*) are the subject of this review.



Figure 1: *Ganoderma lucidum* (A), *Trametes versicolor* (B) (previous *Coriolus versicolor*), *Grifola frondosa* (C), *Hericium erinaceus* (D) and *Cordyceps militaris* (E)

Technological possibilities for commercial cultivation gave rise to the number of patents, which are protecting inventions related to new methods and technologies for cultivation of fruit bodies and/or mycelium biomass, methods of active compounds isolation, and development of new commercial formulations and products. *Ganoderma lucidum* has been identified as a medicinal mushroom with the largest number of patented inventions [11].

#### a) *Ganoderma Lucidum*

*Ganoderma* (W. Curt.: Fr.) Lloyd is a white rot wood *Basidiomycete* that degrades lignin and possess hard fruiting body. *G. lucidum* is from *Ganoderma sp.* the most often reported as a source of various medicinal compounds. In Asian traditional medicine, the fruiting body of *G. lucidum*, named Ling-zhi in Chinese and Reishi in Japanese language, has been used for treatment of several diseases for thousands of years, as

reported in Shen Nong's Materia Medica (Leung et.al., 2002; Kim and Kim, 2002; Lin, 2009).

Modern uses of *Ganoderma* include treatment of coronary heart diseases, arteriosclerosis, hepatitis, arthritis, nephritis, bronchitis, asthma, hypertension, cancer and gastric ulcer [15,16]. Publications also report on *Ganoderma* antiallergenic constituents [17], immunomodulatory action [18-20], antitumor activity [21-23] cardiovascular effects [24], liver protection and detoxification, and effects on nervous system [22,25]. New reports emphasize its potential in treatment of viral, especially HIV infections [26, 27].

Pharmaceutically active compounds from *Ganoderma lucidum* include triterpenoids, polysaccharides (1,6- $\beta$ -D-glucans and 1,3- $\beta$ -D-glucans), proteins, proteoglycans, steroids, alkaloids, nucleotides, lactones and fatty acids, amino acids, nucleotides, alkaloids, steroids, lactones and enzymes. Over 100 triterpenoids were found in *Ganoderma* spp., such as ganoderic (highly oxygenated C<sub>30</sub> lanostane-type triterpenoids), lucidenic, ganodermic, ganoderenic and ganolucidic acids, lucidones, ganoderals and ganoderols [11].

A large and diverse spectrum of chemical compounds with a pharmacological activity has been isolated from the mycelium, fruiting bodies and sclerotia of *Ganoderma* mushrooms: triterpenoids, polysaccharides, proteins, amino acids, nucleotides, alkaloids, steroids, lactones, fatty acids and enzymes [28, 29]. There is abundant evidence that polysaccharides isolated from *G. lucidum* are immunomodulatory effective [4, 30-34,]. Studies have shown that the most active immunomodulatory polysaccharides are (1 $\rightarrow$ 3)- $\beta$ -D and (1 $\rightarrow$ 6)- $\beta$ -D glucans, that can be precipitated by ethanol. Their prevailing structure is  $\beta$ -1,3 D-glucopyranan with 1–15 units of  $\beta$ -1,6 monoglucosyl side chains. Their 1,3-linked backbone, relatively small side chains, and an organized helical structure are beneficial for the immunostimulation. Although they are chemically heterogeneous, these polysaccharides are usually termed as  $\beta$ -glucans [30-32, 36, 37].

Bioactive polysaccharides have been isolated from different sources of *G. lucidum*: basidiocarps, spores and from the mycelial biomass cultivated in liquid culture. Few have been isolated from the culture medium [37]. Though different models of the fungal cell wall differ somewhat, scientists agree that  $\beta$ -glucan is not located on the surface of the wall but is more or less immersed in the wall material. Generally, the cell wall of most fungi contains five main components: (1 $\rightarrow$ 3)- $\beta$ -D glucan, (1 $\rightarrow$ 6)- $\beta$ -D glucan, chitin, and glycoproteins.  $\beta$ -glucan forms 9–46 % of cell wall mass [36, 37]. A large number of studies have shown that polysaccharides of *G. lucidum*, especially  $\beta$ -D glucan, can modulate the functions of many components of the immune system

such as the antigen-presenting cells, T and B lymphocytes, NK cells, neutrophil granulocytes, dendritic cells and, on cytokine production [30, 32, 38, 39].

#### b) *Grifola Frondosa*

*Grifola frondosa* also known as Maitake is white rot lignin degrading *Basidiomycete* with excellent nutritional and medicinal properties. *Grifola frondosa* active compounds primarily belong to the group of polysaccharides (especially 1,6- $\beta$ -D-glucans and 1,3- $\beta$ -D-glucans), glycoproteins, and proteins. These products have been used for treatment of a series of diseases, including hepatitis, arthritis, nephritis, bronchitis, coronary heart diseases, asthma, arteriosclerosis, hypertension, cancer and gastric ulcer. Newer investigations report on *Grifola frondosa* antiallergenic constituents, immunomodulatory action and treatment of HIV infections, antitumor and cardiovascular effects, liver protection and detoxification and effects on nervous system [40].

*G. frondosa* has gained in popularity among consumers, not only because as a gastronomic delight of its taste and flavor, but also because of its reported medicinal value. Its active compounds primarily belong to the group of polysaccharides (especially 1,6- $\beta$ -D-glucans and 1,3- $\beta$ -D-glucans), glycoproteins, and proteins. Medicinal effects of *G. frondosa* are numerous, including anti-cancer activity [41, 42], immune system stimulation [43,44], effects on angiogenesis [45], reduction of benign prostatic hyperplasia [46], antibacterial [47], and antiviral effects [48], effects on lipid metabolism and hypertension [41], antidiabetic activity [49], vitality and performance enhancement [50], antioxidant effects, and beneficial cosmetic effects on skin [51]. According to Shen (2001), more than 20 anti-tumor polysaccharides have been isolated and purified from *G. frondosa* [52].

#### c) *Hericium Erinaceus*

In *H. erinaceus* various pharmaceutically active substances were found. Phytosterols ( $\beta$ -sitosterol and ergosterol), lower the content of low density lipoproteins (LDL) and triglycerides that operate anticarcinogenic as well they reduce the metabolism of fats [53]. In *H. erinaceus* fruitbody numerous constituents such as are polysaccharides, proteins, lectins, phenols, hericenones, erinacines and terpenoids were identified. They strengthen the immune system, relieve gastritis and gastrointestinal infections, reflux and upset stomach due to stress [54].

*H. erinaceus* water-soluble polysaccharides increased activity of macrophages and other immune cells in the fight against cancer cells, but also demonstrated the reduction of formation of metastases. The most outstanding activity of the extract of *H. erinaceus* is that it strengthens the immune system and

activate the synthesis of nerve growth factor [55]. Due to the increased proliferation of T and B-lymphocytes it strengthens the immune system and strengthens the body's natural defences. Thus, *H. erinaceus* expresses very positive effects on prolongation of quality of life of the cancer patients [53].

Among the compounds isolated from fruiting bodies and cultured mycelia of *H. erinaceum*, most interesting are the low-molecular-weight compounds belonging to a group of cyathin diterpenoids (erinacines A–K, P, and Q). Several of them, i.e. erinacines A–H, are known to have a potent stimulating effect on nerve growth factor (NGF) synthesis *in vitro* [56–61].

*H. erinaceus* polysaccharides (HEP) derived from fruiting bodies and mycelium severed as effective therapeutic agents in liver damage-associated diseases. A study demonstrated that the serum levels of aspartate aminotransferase and glutamic pyruvic transaminase activities in carbon tetrachloride-induced hepatic injury were decreased by administration with extracellular and intracellular HEP (200, 400, and 800 mg/kg/day) from mycelium, but the blood lipid levels in the serum of mice were enhanced [62]. Moreover, Kim *et al.* found that 10 mg/kg/day HEP markedly alleviated *Salmonella typhimurium*-induced liver damage and reduced infected mice mortality [63]. Zhang *et al.* revealed that endo-HEP potent hepatoprotective effect *in vivo*, which may be due to its powerful antioxidant capacity. Taken together, the HEP could be exploited as a supplement in the prevention of hepatic diseases [64].

#### d) *Trametes Versicolor*

*Trametes versicolor*, previous *Coriolus versicolor*, also known as Turkey tail mushroom is one of the most attractive medical fungi. It is known for its use and success as a remedy in Asian traditional medicine [65–67]. *T. versicolor* pharmaceutical activities include immunomodulation, antibody production, activation of apoptosis etc.

The two most prominent products of *T. versicolor* are polysaccharide Krestin (PSK) and polysaccharide peptide (PSP) both potentially highly active pharmaceutical substances in complementary cancer treatments. PSP has a variety of physiological effects, such as immunological enhancement, antitumor, liver protecting, oxidation resistance, and reducing blood fat. PSP has been clinically used in treating cancers, hepatitis, hyperlipidemia, chronic bronchitis, and other diseases. The clinical data also demonstrate that PSP has diverse functions such as improving the quality of patients' life, enhancing learning and memory, and antiulcer effects [70–73].

Current studies support PSP as an immunotherapeutic. PSP activates and enhances the function and recognition ability of immune cells, strengthens the phagocytosis of macrophages, increases the expressions of cytokines

and chemokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukins (IL-1 $\beta$  and IL-6), histamine, and prostaglandin E, stimulates the filtration of both dendritic cells and T-cells into tumors, and ameliorates the adverse events associated with chemotherapy. In recent years, immunotherapy has been widely used in cancer treatment. However, to use PSP as an immunotherapeutic at world stage, further chemical, biochemical and pharmacological studies of PSP are needed [66].

*In vitro* and *in vivo* studies have shown that the mixture of PSP and PSK has a synergistic action that highly affects immune cell proliferation and highly expresses antitumor activities [65, 67, 68, 74–76].

#### e) *Cordyceps Militaris*

The usage of natural/herbal medicines over the synthetic ones has seen an upward trend in the recent past. *Cordyceps* being an ancient medicinal mushroom used as a crude drug for the welfare of mankind in old civilization is now a matter of great concern because of its unexplored potentials obtained by various culture techniques and being an excellent source of bioactive metabolites with more than 21 clinically approved benefits on human health [77–78].

The studies by many researchers in the past on *Cordyceps* have demonstrated that it has anti-bacterial, anti-fungal, larvicidal, anti-inflammatory, anti-diabetic, anti-oxidant, anti-tumor, pro-sexual, apoptotic, immunomodulatory, anti-HIV, remarkable clinical health effects including action on hepatic, renal, cardiovascular, respiratory, nervous, besides having anti-cancer, anti-oxidant, anti-inflammatory and anti-microbial activities [79–82].

Cordycepin has received much attention due to its broad-spectrum biological activity. It is known to interfere with various biochemical and molecular processes including purine biosynthesis [83, 84], DNA/RNA synthesis [85] and mTOR (mammalian target of rapamycin) signaling transduction [86]. *Cordyceps* has been included as one of the growing numbers of fungal traditional Chinese medicine (FTCM) used as cures for modern diseases with many products available commercially.

Great potential of pharmaceutical active compounds and *Cordyceps militaris* extract contains many biological bioactive materials, such as the terpenoids cordycepin and cordycepic acid, polysaccharides, sterols and other compounds [87]. *Cordyceps militaris* main active component is terpenoid cordycepin that inhibit the development of cancer cells including antitumor, anti-metastatic, insecticidal, anti-proliferative, anti-bacterial properties, anti-fungal, larvicidal, anti-inflammatory, anti-diabetic, anti-oxidant, pro-sexual, apoptotic, immunomodulatory, anti-HIV, remarkable clinical health effects including action on hepatic, renal, cardiovascular, respiratory, nervous,

besides having anti-cancer, anti-oxidant, anti-inflammatory and anti-microbial activities, anti-leukemia and antimalarial activities [77-82].

The second main active components are polysaccharides, which research have shown to be effective in regulating blood sugar and also have anti-metastatic and antitumor properties [87-89]. The most outstanding active substance of *C. militaris* is Cordycepin. The structure of Cordycepin is very much similar with cellular nucleoside, adenosine. Cordycepin, i.e., 3'-deoxyadenosine, is the main active constituent which is most widely studied for its medicinal value having a broad spectrum biological activity and acts like a nucleoside analogue [90].

Cordycepin alone has been widely explored for its anti-cancer/anti-oxidant activities, thus, holding a strong pharmacological and therapeutic potential to cure many dreadful diseases in future. Further investigations need to be focused on to study the mechanistic insight into the mysterious potential of this medicinal mushroom on human health and promoting

its cultivation strategies for commercialization and ethno-pharmacological use of this wonderful herb [91, 92].

#### IV. CULTIVATION TECHNOLOGIES

Since medicinal mushrooms are scarce in nature, cultivation of fruit bodies on artificial media has been introduced. Traditional cultivation of fruit bodies on wood logs has been known for centuries. With time, cultivation methods have diversified, modified and developed (Figure 2). [11].

Besides on wood logs, fruit bodies are being produced on sawdust substrates in trays or beds, and in sterilised plastic bags or in bottles. In addition, production of fungal mycelia has been developed in bioreactors, utilizing submerged cultivation in liquid media, or solid state cultivation on various secondary wastes substrates from wood and agricultural industry [93].

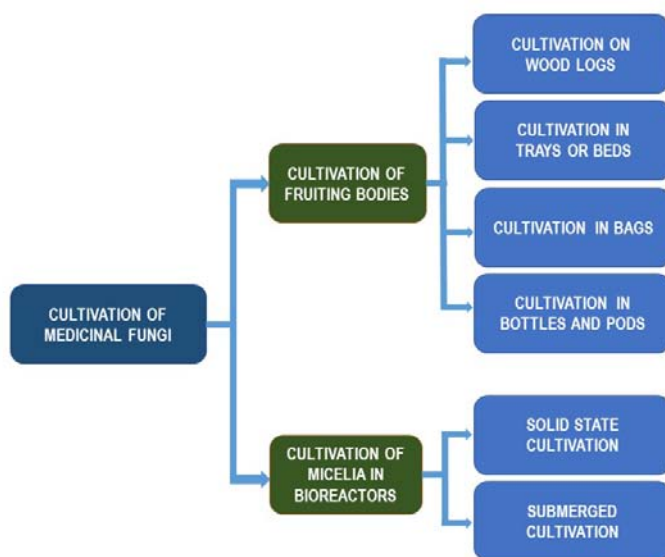


Figure 2: Technological possibilities of cultivating medicinal fungi fruit bodies or fungal biomass on a commercial scale adapted from [11]

##### a) Farming Fruit Bodies Cultivation

In the wild, wood degrading mushrooms grow primarily on hardwood of trees. However, under artificial cultivation conditions, they thrive on various other substrates containing lignin and cellulose, and therefore have a high potential for recycling different types of organic waste materials from wood and agriculture industry. As lignocellulose containing wastes are produced worldwide in large quantities, and in many instances they pose a threat to the environment. Cultivation of edible and medicinal wood degrading fungi on lignocellulosic substrates offers almost unlimited possibilities and economically viable potentials

for large scale commercial cultivation on a World scale [94].

In farming fruit bodies cultivation substrate cultivation methods are divided mostly into bottle cultivation and bag cultivation, sometimes also called synthetic logs. Bag cultivation has more advantages, such as the use of more substrate, a strong body and convenient manipulation, so it is more widely used. Both production processes include the following main steps: raw material preparation, mixing, bagging (bottling) and sterilization, inoculation, spawning, embedding in soil (or transfer to mushroom house), fruiting body development, management and harvesting [94].



Figure 3: Farming production of *Ganoderma lucidum* fruit boddies (Photo A.Gregori )

For a production of *G. lucidum* fruit bodies supplemented sawdust is performed in heat-resistant polypropylene bottles or bags. Sawdust can be supplemented with rice bran (10%) and  $\text{CaCO}_3$  (3%), moistened with water and filled (700 g) into plastic bags. A plastic collar is then fitted onto each bag and stopped with a cotton plug. After 5h of heat treatment (95-100°C) and cooling, substrate is inoculated with grain or sawdust spawn, and incubated for 3 to 4 weeks (or until the spawn fully colonizes the substrate) [95].

#### b) Solid State Cultivation

Solid state cultivation (SSC) is a three-phase heterogeneous process taking place in various bioreactors, comprising solid, liquid and gaseous phases, which offers potential benefits for the microbial cultivation for bioprocesses and products development.

Microbial growth on solid state substrate particles is very close to the growth of fungi in the natural environment. Main source of water, carbohydrates, phosphorous, nitrogen and sulphur are intrapartically bounded, therefore the microbial culture applied have to possess the abilities to access the water and essential element sources out the solid matrix. Concerning that on the tips of young growing hyphae fungal polysaccharides are produced. Polysaccharide gel has actually two functions. Primary it serves as a gel media where from lignocellulitic enzymes are secreted in the solid wooden matrix and secondary as a sticky material used for anchoring of hyphae and for moving on the solid surface. Produced fungal polysaccharides have also a whole palette of their pharmaceutical activities that are used in traditional Eastern medicine already for centuries [96].

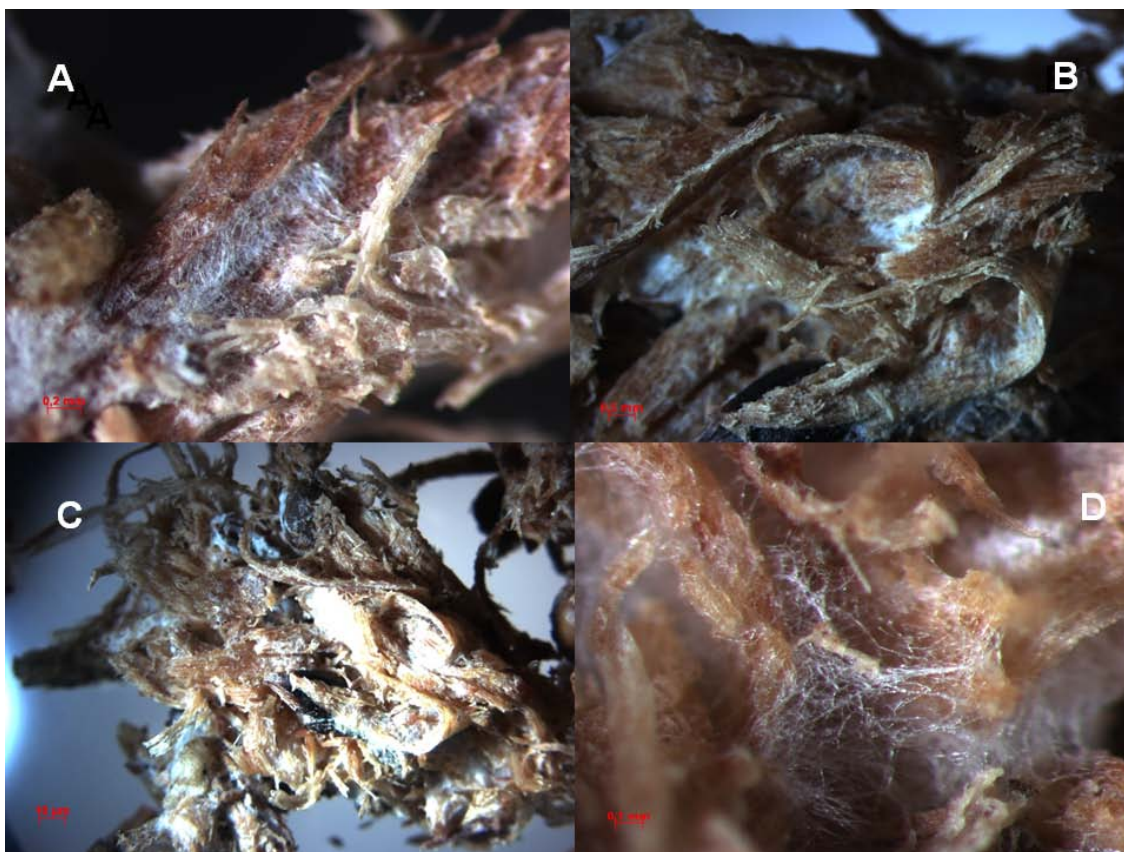


Figure 4: Growth of *Hericium erinaceus* on solid substrate 124 h (D), 145 h (A), 234h (B) and 280 h (C), (Photo M. Vittori)

SSC in bioreactors involves the growth and metabolism of microorganisms in fully control environment. Microbial growth in takes place in aerated beds of moist solid materials in which the interparticle spaces contain a continuous gas phase and little or no free water. The upper limit of moisture content for solid state cultivations is determined by the absorbancy of the solid, which varies between substrates, although for most substrates a free water becomes apparent before 80% moisture level is reached. Although fungal mycelia growth in SSC is very close to the growth in nature habitats fungal fruit boddies are not produced in this technology [93]. An example of SSC mycelia growth in presented in Figure 4.

Over the last two decades, SSC has gained significant attention for the development of industrial

bioprocesses, particularly due to lower energy requirement associated with higher product yields and less wastewater production with lesser risk of bacterial contamination.

An important advantage of solid state cultivation over other techniques is that a concentrated product can be obtained from a cheap substrate, such as wood and agricultural secondary residue with little pretreatment or enrichment. For this reason, solid state cultivation seems to be most appropriate for the production of pharmaceutically active animal feed supplements, for which the whole fermented substrate can be used as the product [97]. Results of solid state medicinal mushroom cultivation on various substrates are presented in Table 1.

Table 1: Solid state cultivation of various medicinal mushroom in 15 L horizontal stirred tank bioreactor (HSTR). Own results

Fungus	Substrate	Biomass (mg/g)	Intracellular IPS (mg/g)
<i>Ganoderma lucidum</i> *	Beech saw-dust	68	7.45
<i>Grifola frondosa</i>	Beech saw-dust	54	4.70
<i>Trametes versicolor</i>	Corn straw	83.5	5.95
<i>Hericium erinaceus</i>	Husked and paddy millet	350	3.07
<i>Cordyceps militaris</i>	Husked paddy millet+ rice	236	10.42

\*European strain MZKI G93



An important advantage of solid state cultivation over other techniques is that a concentrated product can be obtained from a cheap substrate, such as an agricultural residue with little pretreatment or enrichment. On the other hand, the use of an undefined medium, such as sawdust, might make the product purification more difficult. For this reason, solid state cultivation seems to be most appropriate for the production of pharmaceutically active animal feed supplements, for which the whole fermented substrate can be used as the product [98].

In recent years, substantial credibility in employing solid state cultivation (SSC) technique has been witnessed owing to its numerous advantages over

submerged bioprocessing (SC). In spite of enormous advantages, true potential of SSC technology has not yet been fully realized at industrial scale [96].

c) *Submerged Cultivation*

Submerged cultivation of mushrooms represent the best and the fastest technology for a large scale production of medicinal mushroom mycelia and their products for a human use. In recent years, its submerged cultivation has received great interest in Asian countries as a promising alternative for efficient production of medicinal mushroom mycelia and its valuable metabolites [99].

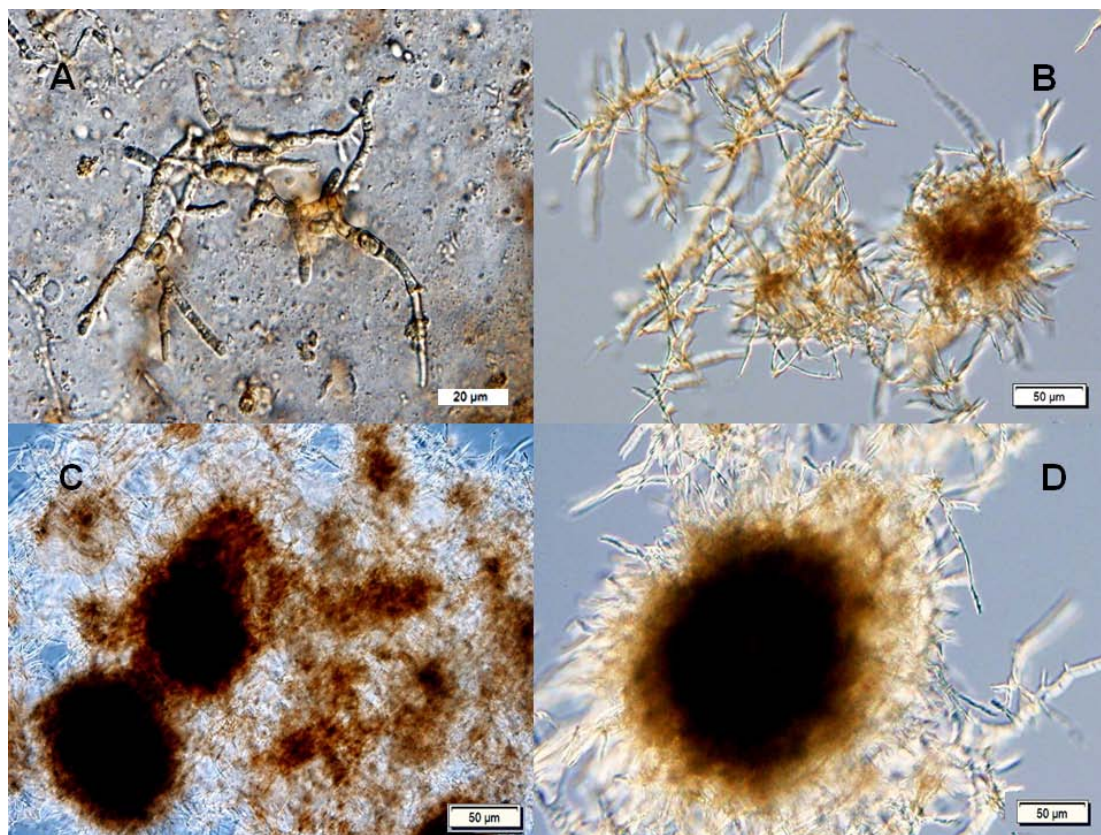


Figure 5: Growth of *Hericium erinaceus* in submerged cultivation. (A) fungal mycelia (14 h)(magnification 1000x); (B) mycelia clumps (218 h) (400x), (C) mycelia pellets (262 h) (100x), (D) mycelia pellets (346 h) (100x) (Photo M.Vittori)

Mycelial growth and the results of submerged medicinal mushroom cultivation of five species on various substrates are presented in Figure 5 and Table 2.

The problems in submerged cultivation of fungal biomass increase with increase the broth viscosity during cultivation because of changes in the morphology, fungal biomass and extracellular polysaccharide (EPS) production. Therefore one of the most important factors of large-scale submerged cultures in bioreactors is related to the heat and mass transfer liquid phase oxygen supply. It is necessary to characterize the variations that occur during the

submerged cultivation in bioreactors and their effects on growth and product formation [100,101].

Table 2: Results of submerged cultivation of five species of medicinal mushrooms in bioreactors

Species	Substrate	Bioreactor	Products	
<i>Grifola frondosa</i>	Optimized medium for mycelial biomass: 45.2 gL <sup>-1</sup> glucose, 2.97 gL <sup>-1</sup> KH <sub>2</sub> PO <sub>4</sub> , 6.58 gL <sup>-1</sup> peptone Optimized medium for extracellular polysaccharides 58.6 gL <sup>-1</sup> glucose, 4.06 gL <sup>-1</sup> KH <sub>2</sub> PO <sub>4</sub> 3.79 gL <sup>-1</sup> peptone	15 L STR bioreactor inoculum 10% (v/v), T 25 °C, initial pH 5.5, aeration rate 8.0 vvm, agitation speed 80 rpm	Biomass 22.50 gL <sup>-1</sup> Extracellular polysaccharides 1.32 gL <sup>-1</sup>	[103]
<i>Ganoderma lucidum</i>	Potato dextrose 101.2 gL <sup>-1</sup> glucose 2 % olive oil	10 L STR bioreactor inoculum 10% (v/v) T 30 °C, initial pH 5.8, aeration rate 1.0 vvm, agitation speed 300 rpm	Biomass 15.9 gL <sup>-1</sup> Extracellular polysaccharides 9.6 gL <sup>-1</sup> Intracellular polysaccharides 6.3 gL <sup>-1</sup>	[104]
<i>Hericium erinaceus</i>	30 gL <sup>-1</sup> corn flour 10 gL <sup>-1</sup> glucose 3.0 gL <sup>-1</sup> yeast extract 1.0 gL <sup>-1</sup> KH <sub>2</sub> PO <sub>4</sub> , 0.5 gL <sup>-1</sup> CaCO <sub>3</sub> 15 mL of corn steep liquor	15 L STR bioreactor inoculum 10% (v/v), T 28 °C initial pH 5.7 aeration rate 0.8 vvm, agitation speed 80 rpm	Biomass 20.5 gL <sup>-1</sup> Extracellular polysaccharides 4.25 gL <sup>-1</sup>	[105]
<i>Trametes versicolor</i>	35 gL <sup>-1</sup> glucose 0.5 gL <sup>-1</sup> yeast extract, 5.0 gL <sup>-1</sup> pepton 1.0 gL <sup>-1</sup> KH <sub>2</sub> PO <sub>4</sub> 0.5 gL <sup>-1</sup> MgSO <sub>4</sub> x 7 H <sub>2</sub> O 0,05 gL <sup>-1</sup> tiamin	10 L STR inoculum 10% (v/v) , T 28 °C initial pH 5.7 aeration rate 1.0 vvm, agitation speed 400 rpm	Biomass 18,5 gL <sup>-1</sup> Extracellular polysaccharide 3.8 gL <sup>-1</sup>	[106]
<i>Cordyceps militaris</i>	80 gL <sup>-1</sup> glucose, 10 gL <sup>-1</sup> yeast extract, 0.5 gL <sup>-1</sup> MgSO <sub>4</sub> ·7H <sub>2</sub> O 0.5 gL <sup>-1</sup> KH <sub>2</sub> PO <sub>4</sub>	5 L STR bioreactor T 24 °C, pH 5.8, agitation 200 rpm, aeration 1.5 vvm,	Biomass 40.60 gL <sup>-1</sup> Extracellular polysaccharide 6.74 gL <sup>-1</sup>	[107]

d) Submerged Cultivation of the other Species in Bioreactors

Besides the cultures present in this chapter, there are also some other medicinal mushrooms that were submerge cultivated in mostly in lab scale bioreactors.

*Inonotus levis* and *Agaricus nevoi* cultivation was proceed in a 10 L stirred-tank bioreactor using substrate based on glucose and corn steep liquor at pH 5.5. Agitation speed gradually increased from 50 to 300 rpm and T 28 °C. *I.levis* developed very rapidly and after 5 days of cultivation the culture reached the stationary phase of growth with a high level of mycelia biomass of 16 g/L at level of EPS concentration 4.2 g/L. *A. nevoi* was distinguished by a much lower growth rate and entered the stationary growth phase on day 10 with mushroom biomass 12 g/L and EPS of 3.9 g/L [102].

Submerged cultivation of *Agaricus brasiliensis* was studied in 1 L stirred tank reactor. Sucrose was found to be most effective for EPS production. Yeast extract was the best for EPS among the inorganic and organic nitrogen sources tested. The factorial experiment demonstrated that a temperature of 30 °C and a pH of 6.1 were the best for the EPS production.

Glucose 10 g/L, yeast extract 3 g/L , K<sub>2</sub>HPO<sub>4</sub> 0.6 g/L and MgSO<sub>4</sub> 0.3 g/L [108].

*Armillaria mellea* was cultivated on glucose 40 g/L and yeast extract based substrate in 5 L stirred tank reactor at 22°C; the two-stage aeration rate strategy (1.2→0.6 vvm); 150 rpm, controlled pH 4.0, 6.65 g/L fungal biomass and 233.2 m g/L of extracellular polysaccharides with antioxidant properties were obtained [109].

*Pleurotus pulmonarius* was studied in submerged cultivation in a 2 L stirred-tank reactor. Substrate was composed by 20 g/L of brown sugar, 4 g/L rice bran, 4 g/L malt extract, and 4 g/L of yeast extract with initial pH of 5.5 Incubated at 28°C with agitation speed of 250 rpm and oxygen partial pressure of 30–40%. Maximum *P. pulmonarius* dry biomass production of 11.8 g/L was achieved after 3 days of cultivation [110].

*Pleurotus saca* was submerged cultivated on substrate consist of beer worth substrate (batch mode in 10-L stirred tank reactor. Agitation speed was 500 rpm and aeration 5 Lmin<sup>-1</sup> and pH 6.2, up to 48.5 g/L of dry biomass was obtained [111].

*Pleurotus ostreatus* was cultivated in a 20 L stirred tank bioreactor in a submerged process with enhanced glucan and dietary fibres content, using 57 g/L xylose and 37 g/L corn steep liquor. High yields 39.2 g/L of dry biomass was obtained [112,113].

*Ganoderma lingzhi* were studied in 5 L stirred bioreactor. The optimum conditions were an initial pH of 5.9, 20.0% DO and T 29° C. These conditions resulted in a triterpene acids (TA) of 0.31 g/L. Furthermore this optimized conditions were then successfully scaled up

to a production scale of 200 L, and maximum TA production and productivity of 0.29 g/L and 0.05 g/L day<sup>-1</sup> were achieved. [114].

e) *Differences between Solid State and Submerged Cultivation*

Main difference and benefits of solid state and submerged medicinal mushroom cultivation are presented in Table 3.

Table 3: Comparison of solid-state and submerged cultivation [96]

Solid state cultivation	Submerged cultivation
Some products can only be produced well under low moisture conditions. For other products, if the producing organisms require free water, solid state cultivation cannot be used.	A wide range of products can be produced, from a wide range of microorganisms and fungi. Many products are produced best under submerged cultivation.
The medium is relatively simple (eg. grain) and unrefined. It may contain all nutrients necessary for growth, or only require wetting with a mineral solution. Pretreatment can be as simple as cooking or grinding. However, the substrate composition and characteristics can be variable.	The medium often contains more highly processed ingredients and is therefore more expensive. Unprocessed ingredients may need processing to extract and solubilize the nutrients. With defined media good reproducibility is possible
The low water availability helps to select and protect against growth of contaminants.	The water activity is usually very high and many contaminants can grow well.
Media are concentrated and smaller bioreactors can be used, leading to higher volumetric productivities, even when growth rates and yields are lower.	Media are dilute and therefore occupy larger volumes, leading to lower volumetric productivities.
High substrate concentrations can enable high product concentrations.	High substrate concentrations can cause rheological problems. Substrate feeding systems may be required.
Aeration requires less power since pressures are lower. Gas transfer is easier since the particles have a large surface area.	High air pressures can be required. Gas transfer from the gas to liquid phase is slow and can be limiting.
Mixing within particles is not possible, and growth can be limited by the diffusion of nutrients.	Vigorous mixing can be used, and diffusion of nutrients is usually not limiting.
Ability to remove metabolic heat is restricted, leading to overheating problems.	High water content and more dilute nature makes temperature control easier.
Process control can be difficult due to difficulties in making on-line measurements, and in measuring biomass. The addition of substances during the process is difficult.	Many on-line sensors are available and more are being developed. Additions of substances can be made to control the process.
Downstream processing may be simpler since products are more concentrated. However, extracts can be contaminated with substrate components.	Downstream processing requires removal of large volumes of water, and is more expensive. However, with defined media, product purification may be easier.
Liquid waste is not produced.	Usually large volumes of liquid wastes are produced.
Growth kinetics and transport phenomena have received little attention and are poorly characterized.	Much kinetic and transport information is available in literature, which can guide reactor design and operation.
Research results and information from the solid-state cultivation can be scaled-up, or even transferred and applied in liquid-state cultivation.	In scaling up fungal submerged cultivation processes, various technical problems need to be solved, such as an increased broth viscosity and oxygen supply.
Solid-state cultivation of fungal mycelia is less labor intensive.	Submerged cultivation is more demanding and labor intensive.

## V. DOWN STREAM PROCESSING

Disruption of medicinal mushrooms by mechanical, chemical or enzymatic methods is greatly required for the efficient extraction of active compounds from them. In addition, ultrafine powder of medicinal mushroom by mechanical method can be used for functional food or dietary supplement. Other products used for pharmaceuticals are produced at the stages of extraction, fractionation and purification by varying techniques including microwave assisted extraction, membrane separation, adsorptive separation and chromatography. Recrystallization, lyophilisation, drying and formulation are used as final product treatments [115].

Current disruption methods can be classified into mechanical, chemical and enzymatic methods in terms of their principles and characteristics. Mechanical methods are often preferred due to short residence time and lower operating costs [116]. The most common mechanical means for disruption are bead mill and homogenizer [117]. Another most frequently used sample disruption method is air jet milling which uses high velocity jets of gas to impart energy to particles for size reduction. The main features of air jet mills include [118].

Extraction is the first step to separate the desired products from the raw materials. Nearly 80–85 % of all medicinal mushroom products are extracted from their fruiting bodies while only 15 % are derived from mycelium culture [119].

Solvent is one of the most important parameters for a successful extraction. Selectivity, solubility, cost and safety should be considered in selection of solvents. Alcohols (ethanol and methanol) are universal solvents for the extraction of natural products although their low selectivity.

In recent years, advanced and greener extraction methods such as supercritical fluid extraction, pressurized liquid extraction, ultrasound assisted extraction, microwave assisted extraction, pulsed electric field extraction and enzyme assisted extraction have also been applied for extraction of natural products, and they offer some advantages such as lower organic solvent consumption, shorter extraction time and higher selectivity. In particular, supercritical fluid extraction gains increasing attention due to its higher efficiency and greener characteristics.

A brief summary of the various extraction methods used for medicinal mushroom products is shown in Table 4 [120].

*Table 4:* A brief summary of various extraction method used for medicinal mushroom product adapted from [120]

Applied Method	Solvent	Volume of consumed solvent	Temperature	Time	Pressure	Polarity of extracted products
Maceration	Various solvent	Large	Room temperature	Atmospheric	Long	Dependent on the solvent
Percolation	Various solvent	Large	Room temperature, sometimes under heat	Atmospheric	Long	Dependent on the solvent
Decoration	Water	None	Under heat	Atmospheric	Moderate	Polar compounds
Reflux extraction	Various solvent	Moderate	Under heat	Atmospheric	Moderate	Dependent on the solvent
Soxhlet extraction	Organic solvent	Moderate	Under heat	Atmospheric	Long	Dependent on the solvent
Pressurized liquid extraction	Organic solvent	Small	Under heat	High	Short	Dependent on the solvent
Supercritical fluid extraction	Supercritical fluid (usually CO <sub>2</sub> )	None or small	Near room temperature	High	Short	None polar or moderate polar
Ultrasound assisted extraction	Various solvent	Moderate	Room temperature or under heat	Atmospheric	Short	Compounds dependent on the solvent
Microwave assisted extraction	Various solvent	Moderate	Room temperature or under heat	Atmospheric	Short	Dependent on the solvent
Pulsed electric field extraction	Various solvent	Moderate	Room temperature or under heat	Atmospheric	Short	Dependent on the solvent

Enzyme assisted extraction	Various solvent	Moderate	Room temperature or under heat	Atmospheric	Moderate	Dependent on the solvent
Distillation	Water	Moderate	Under heat	Atmospheric	Long	Essential oil

The obtained fraction containing the desired products via fractionation steps described above may consist of several compounds with highly similar chemical and physical properties. Sometimes these compounds are analogues or even isomers as already stated, presenting a huge challenge for the purification. Column chromatography packed with separation media with small particle size is the most prevailing technique to implement the task, because it can offer very high number of theoretical plates and thus high resolution. However, in industrial scale, the particle size of the packed material is much larger because no pump can generate the pressure in manufacturing scale as that in a HPLC system [120].

## VI. CONCLUSION

Various pharmaceutically active substances from medicinal mushrooms represent effective potential in human life. Great demand for medicinal fungi biomass production could be fulfilled using various cultivation techniques. Medicinal fungi biomass in a present time is mostly covered by farming. Farming cultivation represents cheap but long time consuming technology. Using cultivation on a wooden locks a few year coughing time is need. Cultivation on sawdust substrates in trays or beds and in sterilised plastic bags or in bottles represents an advance and much faster production of fungal fruit boddies than conventional farming.

Solid state a few week time cultivation is a comprehensive, well controlled technology that enables much faster medium scale technology for medicinal mushroom mycelia production. In this technology various secondary waste from wood and agriculture industry are successfully used. No fungal fruit boddies are produced. Final product delignolized, wooden material is overgrown by medicinal fungi biomass in two to four weeks, enreach with proteins and various pharmaceutically products need to be dried and pulverized and in such form it could be directly used in a veterinary need. Solid state cultivation of medicinal mushroom biomass is cheap in non time consuming technology perfectly suitable in veterinary use.

Submerged cultivation of medicinal fungi biomass represents fast and comprehensive technology method. Fungal biomass is in its final state from 10 to 28 days. The main benefit of cultivation of medicinal mushroom biomass in bioreactors is in using of higher sterility, comprehensive technology and bioprocess control, for large scale production of various pharmaceutically active compounds as are fungal

polysaccharides, terpenoides or proteinoglucans in much shorter cultivation time. Reports on pharmacological activity of extracts, partly purified preparations and isolated compounds from biomass of *G.lucidum*, *G.fruondosa*, *T.versicolor*, *H.erinaceus* and *C.militaris* and the other reported species in laboratory and pilot scale, are very convincing. Production of medicinal fungi polysaccharides enhanced by fed-batch or two-stage cultivation strategy was found very useful for improving the production. Some of present results of lab scale research of various medicinal fungi are already transferred to pilot and lower industrial scale and they represents suitable starting platform for development of medicinal fungi biomass in large scale pharmaceutical industrial production. Comparing the economy of the same product production solid state processing is 30 % less than those of the one produced in submerged cultivation.

Isolation of medicinal fungi pharmaceutically compounds in all three technologies is based on precipitation with hot water and ethanol. Crude extracts often show equal or stronger pharmacological activity as purified compounds, which suggests potential synergistic effects of several naturally occurring compounds. In the future, more precise capture (or enrichment) and separation techniques such as affinity separation, and more integrated bioprocesses for medicinal mushroom products should be developed which would enable a higher product yield and better process performance.

As pointed out submerged cultivation of medicinal mushrooms has significant large scale industrial potential, but its success on a commercial scale depends on existing field-cultivation technology as well as pharmaceutical market economy. Production of various medicinal mushrooms products, terpenoids, polysaccharides and proteinoglucans represents great business in Asiatic space where it is traditional. Great demand of this active ingredients definitively need to include new and fast large scale industrial production technologies as are solid state and submerged cultivations. In opposite, Western Pharmaceutical Industry has no tradition in natural isolates from herbal and fungal sources. Unfortunately, it is too convinient and much based on classical pharmaco-chemical biosynthesis including its all side effects.

However, from the viewpoint of Western Science, pharmaceutical legislation and regulations might be one of the main obstacles hindering the introduction of medicinal mushrooms products as registered pharmaceuticals. In any case, further

research is needed to fully understand all mechanisms of pharmaceutical effects of medicinal mushrooms products and to identify their potential side effects.

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