Review

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Mushroom immunomodulators: unique molecules with unlimited applications

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For centuries, mushrooms have been used as food and medicine in different cultures. More recently, many bioactive compounds have been isolated from different types of mushrooms. Among these, immunomodulators have gained much interest based on the increasing growth of the immunotherapy sector. Mushroom immunomodulators are classified under four categories based on their chemical nature as: lectins, terpenoids, proteins, and polysaccharides. These compounds are produced naturally in mushrooms cultivated in greenhouses. For effective industrial production, cultivation is carried out in submerged culture to increase the bioactive compound yield, decrease the production time, and reduce the cost of downstream processing. This review provides a comprehensive overview on mushroom immunomodulators in terms of chemistry, industrial production, and applications in medical and nonmedical sectors.

Introduction

Immunomodulators are key components in the modern health and wellness industries, reflecting the fact that the immune system is the first barrier for disease prevention. In any healthy organism, the immune system produces a wide range of immunomodulators to maintain homeostasis within the body. In clinical practice, immunomodulators are usually classified into immunosuppressants, immunostimulants, and immunoadjuvants (see Glossary). Their market share has increased rapidly over the past few years due to wide-ranging medical applications for stimulation and suppression of the immune system. They are even used as prodrugs or prophylactic medicine for healthy people. The market size of immunomodulators is valued at $145.9 billion in 2012 and is proposed to increase rapidly with a compound annual growth rate of 8.6% reaching $259.3 billion in 2017 [1]. Chemically synthesized compounds and monoclonal antibodies of antiproliferative and antimetabolic drugs generate the highest revenues among all classes of immunomodulators. Recently, there has been growing interest in natural immunomodulators as alternatives to the currently used chemical drugs that have a high-risk profile [2]. Mushrooms are among the most interesting natural sources of compounds for pharmaceutical applications and are central components of traditional medicine worldwide. This is because they show greater diversity and possess unique bioactivity compared with other natural sources.

Mushroom polysaccharides such as lentian, schizophyllan, polysaccharide K (Kerstin), and polysaccharide peptide (PSP) are now available on the pharmaceutical market. For example, lentinan is applied as adjuvant cancer immunotherapy or in parallel to radio- and chemothermotherapy [3].

Mushrooms: nutritional and medicinal facts

Mushrooms can be defined as macrofungi with distinctive fruiting bodies that are either epigeous (of fruiting bodies above the ground) or hypogeous (of underground fruiting bodies) and sufficiently conspicuous to the naked eye to be

Glossary

Fruiting body: is a multicellular structure on which spores producing structure as basidia or asci are born. They are distinct in size, shape, and coloration for each mushroom species.
Immunoadjuvants: are used to enhance vaccine efficacy. They can also be described as specific immune stimulators.
Immunomodulators: also known as biological response modifiers (BRMs), immunoaugmentors, or immunorestoratives, are substances, of biological or synthetic origin, which can stimulate, suppress or modulate any of the components of the immune system including both innate and adaptive arms of the immune response.
Immunostimulants: are agents that stimulate the immune system through induction or activation of immune system components or mediators. They enhance resistance against infection, allergy, cancer, and autoimmunity.
Immunosuppressants: are agents that inhibit the immune system. They can be used to control a pathological immune response after organ transplantation and to treat autoimmune diseases, hypersensitive immune reaction, and immunopathology associated with infections.
Immunotherapy: treatment (e.g., using drugs) that induces, enhances, or suppresses an immune response.
Mycelium: is the vegetative part of a fungus, consisting of a mass of branched hyphae. It forms the mushroom stalk in case of solid substrate grown mushrooms, or a complex shape of intertwined thread in submerged culture.
Nutraceuticals: food or part of food that provides medical or health benefits, including the prevention and treatment of disease.

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picked by hand [4]. Approximately 15 000 mushroom species have been identified worldwide, of which only about 650 species are reported to be of medicinal value [5]. Beside their high nutritional value, mushrooms are rich in many bioactive metabolites of high medicinal values such as lectins, polysaccharides, phenolic and polyphenolics, terpenoids, ergosterols, and volatile organic compounds [6]. Mushroom extracts have been shown to exhibit immumomodulator, antitumor/anticancer, antibacterial and antiviral, antioxidant, and antihypoglycemic activities, and as active medicine in the prevention of cardiovascular diseases by their effects as antiatherosclerosis agents [7,8]. Most medicinal mushroom research has been based on tests with crude extracts of the whole mushroom fruiting bodies or mycelia, or with partially purified bioactive compound.

Mushroom immunomodulators

More than 50 known mushrooms harbor immune-regulating organic compounds of highly diversified molecular weight and structure. Box 1 lists the top 10 mushrooms with immunomodulator activities. Mushrooms immunomodulators exhibit stimulating activities for both innate and adaptive immune systems. They proliferate and activate innate immune system components such as natural killer (NK) cells, neutrophils, and macrophages, and stimulate cytokines expression and secretion. These cytokines in turn activate adaptive immunity through the promotion of B cells for antibodies production and stimulation of T cell differentiation to T helper (Th) 1 and Th2 cells, which mediate cell and humoral immunities, respectively [9]. Based on their high molecular weight, mushroom polysaccharides are not able to penetrate the immune cells to activate immune cells directly. Thus, the stimulation mechanism of polysaccharides involves different cell receptors such as dectin-1, Complement receptor 3 (CR3), Lactosylceramide (LacCer), and Toll-like receptor (TLR)2. In such cases, the effectiveness of polysaccharides is governed by their binding affinity to immune cell receptors [10].

In general, mushroom immunomodulators are classified into four main groups: (i) immunomodulatory lectins; (ii) immunomodulatory terpenes and terpenoids; (iii) fungal immunomodulatory proteins (FIPs); and (iv) immunomodulatory polysaccharides. Each class is described below.

Box 1. Top 10 mushrooms with immunomodulatory activity

1. *Agaricus subrufescens* (syn. *A. blazei* or *A. brasiliensis*). This edible mushroom has different common names [Almond portobello, Royal sun agaricus, Princess matsutake (JP)]. It is characterized by almond fragrance and a sweet taste. It contains immunostimulatory compounds such as β-1,3-D-glucans, glucomannan, and proteoglycans.
2. *Cordyceps sinensis*: (syn. *Ophiocordyceps sinensis* or *Cephalosporium sinensis*) has been known for a thousand years as a medicinal mushroom in China and Taiwan. These pathogenic fungi are endoparasitic in arthropods. The immunomodulatory activity of this mushroom is due to the presence of polysaccharides and other compound such as cordycepin.
3. *Ganoderma lucidum*: Common names: Reishi (JP), Ligzhi (CN), spirit plant. It has a long history in Japanese and Chinese traditional medicine. This mushroom is rich with more than 50 types of polysaccharides and peptide-polysaccharides complexes in addition to about 120 bioactive compounds (mainly triterpenes). Most of these compounds can act as strong immunostimulants.
4. *Grifola frondosa*: Common name: Maitake. This mushroom is rich in β-glucan, which activates macrophages, NK cells and lymphokines. It also includes compounds such as ergosterol peroxide (EPO) and lipopolysaccharide (LPS) with significant immunomodulating activities.
5. *Hericium erinaceus*: Common names: Lion's Mane Mushroom, Bearded Tooth Mushroom, and Monkey Head Mushroom. It acts as an immunostimulant based on its content of bioactive polysaccharides of β-1,3-branched-β-1,2-mannan structure.
6. *Inonotus obliquus*: Common name: Chaga. This mushroom is a plant pathogen that grows on the trunk of birch trees in cold regions such as North America, Siberia, and Korea. The immunomodulating activity of this mushroom is based on its high content of β-glucans.
7. *Lentinula edodes*: Common name: Shiitake. This is an edible Japanese mushroom, famous for its immunomodulating properties. It is rich in a special type of polysaccharide called lentinan (1-3-β-D-glucan with 1-6-β-D-glucopyranoside branches).
8. *Pleurotus ostreatus*: Common name: Oyster mushroom. It is widely cultivated in different parts of the world. This mushroom includes different types of polysaccharides that belong to the β-glucan, heteroglycan, and proteoglycan families. Pleuran β-(1-3/1-6)-D-glucan is the most effective and well studied polysaccharide in this mushroom.
9. *Poria cocos*: Common names: Fu Ling (CN), Tuckahoe (US). This mushroom, used in traditional medicine, possesses immune stimulatory activity due to a special type of FIP, and *Poria* cocos protein (PCP).
10. *Trametes versicolor*: Common name: Turkey Tail. It has strong immunomodulatory effects due to a polysaccharide called Krestin (PSK), and also contains an FIP named tvc.

Lectins

Lectins form a diverse group of carbohydrate-binding proteins with specific binding capacities. They have been isolated from different organisms. However, those derived from mushrooms are characterized by specific immunomodulatory, antiproliferative, and antitumor activities. Lectins isolated from *Volvariella volacea* (straw mushroom) exhibit stronger immunomodulatory activity than other known lectins such as concanavalin A [11]. Two lectins extracted from *Tricholoma mongolicum*, TML-1 and TML-2, show immunomodulatory and antitumor activities when tested *in vivo* but not in *in vitro*, and they mediate their action through activation of the immune system rather than any direct cytotoxic effects [12]. These lectins stimulate the production of nitrite and tumor necrosis factor (TNF)-α, and inhibit the growth of mouse lymphoblast-like (p815) mastocytoma cells by the production of macrophage-activating factors. These factors include interferon (IFN)-γ and other cytokines, activated through upregulation of inducible nitric oxide synthase (NOS), interleukin (IL)-1β, and transforming growth factor-β [13].

Lectins derived from the fruiting body of *Grifola frondosa* are characterized by their potent cytotoxic effects, even at very low concentrations, in HeLa cells. An 18-kDa thermostable lectin was isolated from *Ganoderma capense* with higher potent mitogenic activity than concanavalin A toward mouse splenocytes. It also showed antiproliferative activities on leukemia and hepatoma cells [14]. Recently, a novel 15.9-kDa homodimeric, lactose-binding, ricin-B-like lectin (CNL) was purified from the basidiomycete *Clitocybe nebularis* and exhibited antiproliferative activity against

human leukemic T cells [15]. This lectin induces the maturation and activation of dendritic cells (DCs) and thus stimulates several proinflammatory cytokines such as IL-6, IL-8, and TNF-α [16]. The CNL encoding gene has been cloned and successfully expressed in *Escherichia coli* [17].

Terpenes and terpenoids

Terpenes are a large and diversified group of organic compounds consisting of isoprene five-carbon atom units of molecular formula $(C_5H_6)_n$ as building blocks. The terpene compounds are named based on the number of repeated units of terpene building blocks, for example, monoterpenes (10 carbons), sesquiterpenes (15 carbons), diterpenes (20 carbons), sestererpenes (25 carbons), triterpenes (30 carbons), and tetraterpenes (40 carbons). These compounds exist widely in plants as the main components of resin and essential oil. In macrofungi, terpenes are present in modified form (terpenoids or isoprenoids) and show biological activities with potential medical applications. Mushrooms belonging to *Ganoderma* sp., such as *Ganoderma lucidum* and *Ganoderma applanatum*, are known for their high content of triterpenoids, for example, lanostane, which shows immumomodulating and anti-infective activities [18]. The solvent extract of the fruiting body of this basidiomycete contains a wide range of terpenes and terpene derivatives such as ganodermic and ganoderic acids, ganoderals, ganoderols, ganodermanontriol, lucidone, and ganodermanondiol (Figure 1), which exhibit biological activities including stimulation of the nuclear factor (NF)-κB pathway and mitogen-activated protein kinases [19]. Recent research has shown that some *G. lucidum* terpenes can prevent drug nephrotoxicity and inflammation, suggesting that they may have pharmacological applications [20].

FIPs

In recent years, mushrooms have been reported to produce a new family of protein immunomodulators, termed FIPs. Since the discovery of the first FIP (Ling-Zhi-8 from *G. lucidum*) in 1989 [21], 11 different types of FIPs have been isolated. These include FIP-fve from *Flammulina velutipes* (Gr.) Sing [22]; Ling-Zhi-8 (FIP-LZ-8 or FIP-glu) from *G. lucidum* [23]; FIP-gts from *Ganoderma tsugae* Murr [24]; FIP-gsi from *Ganoderma sinensis* [25]; FIP-pcp from *Poria cocos* (Schw.) Wolf [26]; and FIP-vvo and FIP-vvl from *Volvariella volvacea* (Bull.; Fr.) Sing [27]; FIP-aca from *Antrodia camphorate* [28]; FIP-gja from *Ganoderma japonicum*; FIP-gmi from *Ganoderma microsporum* [29]; in addition to one new FIP named FIP-tvc from *Trametes versicolor* [30]. These proteins are grouped together in one family based on highly similar amino acid sequences. They exist as dimers in a dumbbell-shaped structure similar to that of the variable region of immunoglobulin heavy chains, but they exhibit diverse activities (Table 1). The best-known FIP-LZ-8 protein consists of 110 amino acid residues and acts as an immunosuppressive agent. It has also been shown to suppress autoimmune diabetic reactions in an animal model and to increase graft survival in transplanted allogenic mouse skin, with fewer nephrotoxic effects compared to other immunosuppressive agents such as cyclosporine A [31].

The low yield of FIPs extracted from their native mushroom producer strain is a major limitation. Therefore, techniques are being developed for the production of recombinant FIPs in other organisms such as the yeast *Pichia pastoris* and the bacterium *E. coli*. Expression of the LZ-8 gene of *G. lucidum* in *P. pastoris*, for example, leads to the production of a recombinant LZ-8 protein (rLZ-8), which, although lacking the carbohydrate moiety of the native protein, shows the same bioactivity for IL-2 induction as the native protein [32]. The gene encoding FIP-fve protein is also expressed successfully in *E. coli* and the recombinant product exhibits activity similar to the native protein, inducing IL-2 and IFN-γ production [33]. More recently, two shuffled gene libraries were created for the genes *FIP-glu, FIP-gsi, FIP-fve, FIP-vvo* and the mutants *FIP-SN* and *FIP-SJ*, which were successfully expressed in *E. coli*. The recombinant FIPs showed higher immunomodulatory activities compared to those originally produced in mushrooms [34]. The shuffled FIPs, like the native proteins, induced the expression of specific cytokines (IL-2, IL-4, IFN-γ, IL-2 receptor in mouse splenocytes) but not TNF-α.

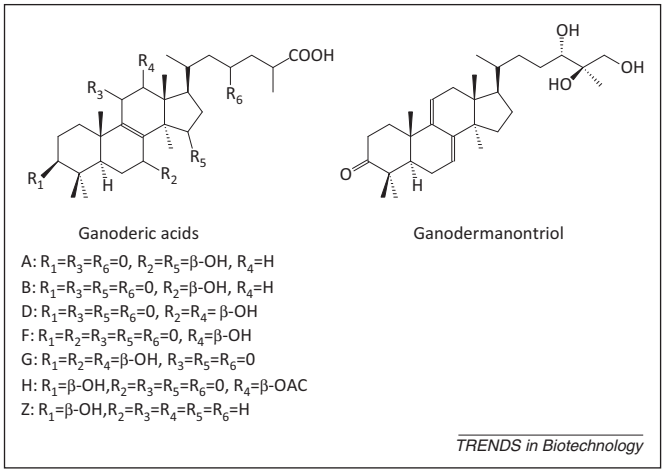

Figure 1. Mushroom triterpenes with immunomodulating activity. The immunomodulatory properties of ganoderic acid and ganodermanontriol are mediated through the activation of natural killer cells and enhancement of interleukin-2 and interferon-γ expression.

Polysaccharides

Mushrooms are an important source of different types of polysaccharides with immunomodulating activities [35]. Most of these polysaccharides are homoglycans (polysaccharides that contain residues of only one type of monosaccharide molecules) or heteroglycans (polysaccharides that contain residues of two or more types of monosaccharide molecules), and are able to combine with other proteins to make peptidoglycan or polysaccharide–protein complexes. The first reported polysaccharide with potential immunomodulating and anticancer activity was lentinan, a β-1,3-D-glucan with β-1,6 branches. (Figure 2). This polysaccharide with triple helical structure was first isolated from the fruiting body of *Lentinus edodes* in the late 1960s in Japan [36]. Since then there have been many research efforts to discover new polysaccharide compounds with immunomodulating activities from mushroom fruiting body extracts. Until the late 1980s, only two more

Table 1. Some FIPs and their immunomodulatory activities

Name of FIP	Producer organism	Immunomodulatory effect	Refs
FIP-aca	*Antrodia camphorate*	Induction of mRNA expression of different cytokines (IL-1β, IL-6, IL-12, TNF-α) and chemokines (CCL3, CCL4, CCL5, CCL10)	[28]
FIP-fve	*Flammulina velutipes*	Stimulation of mitogenesis in human peripheral lymphocytes, suppression of systemic anaphylaxis reaction, enhanced transcription of IL-3, INF-γ	[22]
FIP-glu	*Ganoderma lucidum*	Enhanced transcription of IL-2, IL-3, IL-4, IFN-γ, TNF-α	[23]
FIP-gmi	*Ganoderma microsporum*	Down regulation of TNF-α	[29]
FIP-gts	*Ganoderma tsugae*	Induction of cytokine secretion, cellular proliferation of human peripheral mononuclear cells (HPBMCs), enhanced IFN-γ expression	[24]
FIP-gsi	*Ganoderma sinensis*	Enhanced production of IL-2, IL-3, IL-4, INF-γ, TNF-α	[25]
FIP-pcp	*Poria cocos*	Enhanced production of IL-1β, IL-6, IL-18, TNF-α, NO	[26]
FIP-tve	*Trametes versicolor*	Increased human peripheral blood lymphocytes, enhanced production of TNF-α, NO	[30]
FIP-vvo	*Volvariella volvacea*	Enhanced expression of IL-2, IL-4, IFN-γ, TNF-α	[27]

Abbreviation: CCL, chemokine CC ligand.

polysaccharides of β-glucan type, schizophyllan from *Schizophyllum commune* and the protein-bound polysaccharide Krestin from *Coriolus versicolor*, were characterized with fully proven chemical structure and activities [35]. They were successfully introduced to the nutraceutical and pharmaceutical market as biological response modifier (BRM) drugs based on their ability to restore or enhance immune responses in both *in vitro* and *in vivo* models. However, our understanding of the immunomodulatory effects of mushroom extracts in terms of chemical structure, and exact effects on the immune system remains incomplete.

Table 2 provides a short list of immunomodulator polysaccharides and polysaccharide–proteins of mushroom origin. In general, mushroom polysaccharides are highly diversified in their sugar composition, main chain polymer structure, degree of branching, conformation, molecular weight, and other physical properties, which together have significant effects on the bioactivity and mode of action of the polysaccharide, as discussed below [37].

Effect of molecular weight

In general, high molecular weight polysaccharides usually exhibit higher bioactivity. For example, in a (1→3)-β-glucan extract of *Gr. frondosa* containing different molecular weight fractions, the highest immunomodulatory activity was ascribed to the fraction of 800 kDa and above [38]. In another study, when the polysaccharide krestin (PSK) was fractionated by ultrafiltration, the highest immunomodulatory activity was associated with the highest molecular weight fraction of >200 kDa [39]. By contrast, a low molecular weight 5-kDa fraction of hydrolyzed scleroglucan (a branched β-glucan from *Sclerotium rolfsii*) exhibited strong immunostimulatory activity, increasing the secretion of TNF-α and stimulating the proliferation of lymphocytes [40]. Low molecular weight polysaccharides can penetrate immune cells and exert stimulatory effects. However, the superiority of high molecular weight polysaccharides (which are not able to penetrate the cells) was not clearly discussed in the literature. The superior activity of high molecular weight polysaccharides may be attributed to the better binding affinity to the carbohydrate receptors of the immune cells.

Effect of branching

In most cases, the bioactive immunomodulator polysaccharides are characterized by a main chain of 1-3,-β-D-glucan with a small number of short branched chains with 1-6,β-linkage. The most active polymers generally have a degree of branching number (DB) between 20% and 33%. For example, lentinan (DB = 40% or 2/5) and schizophyllan (DB = 33% or 1/3) are both 1-3,-β-D-glucans with one and two branches for every 5-D-glucopyranosyl and 3-D-glucopyranosyl residue, respectively. The polysaccharide moiety of PSK (DB = 20% or 1/5) is composed of (1-3)-β-/(1-4)-β-D-glucan with one branch for every 5-D-glucopyranosyl residue. Even complete debranching of polysaccharides sometimes increases their bioactivity, for example, pachyman, a branched 1-3,-β-D-glucan of the brown rot fungus *Poria cocos* is inactive, whereas the debranched form, pachymaran, obtained after mild selective periodate oxidation, shows significant activity [41].

Debranching of lentinan also increases its biological activity as immunostimulant and antitumor agent [42]. More recently, a study of the correlation between branching and bioactivity of lentinan showed that among different polysaccharides fractions with DB ranging between 19 and 50%, the maximal immunomodulating and antitumor activities were achieved when the molecule had a DB of 32% [43]. Therefore, we can conclude that the relation between branching and polysaccharides immunomodulatory activity is case specific and needs further investigation.

Effect of helical conformation

Mushroom polysaccharides can exist as single or triple helical forms, as well as random coiled structures. The triple helix conformation is usually more stable than the other structures and bears the cytokine stimulating activity of the β-D-glucan. The most bioactive mushroom polysaccharides known, lentinan, schizophyllan, scleroglucan, and the glucan moiety of PSK, all have a triple helix structure [44]. Furthermore, among different polysaccharide fractions isolated from *Hericium erinaceus*, a low molecular weight fraction with triple helix conformation of a β-1,3-branched-β-1,6-glucan exhibited immunostimulating activity that included macrophage activation, NO production, and expression of IL-1β and TNF-α [45]. On the contrary, other studies have shown that mushroom

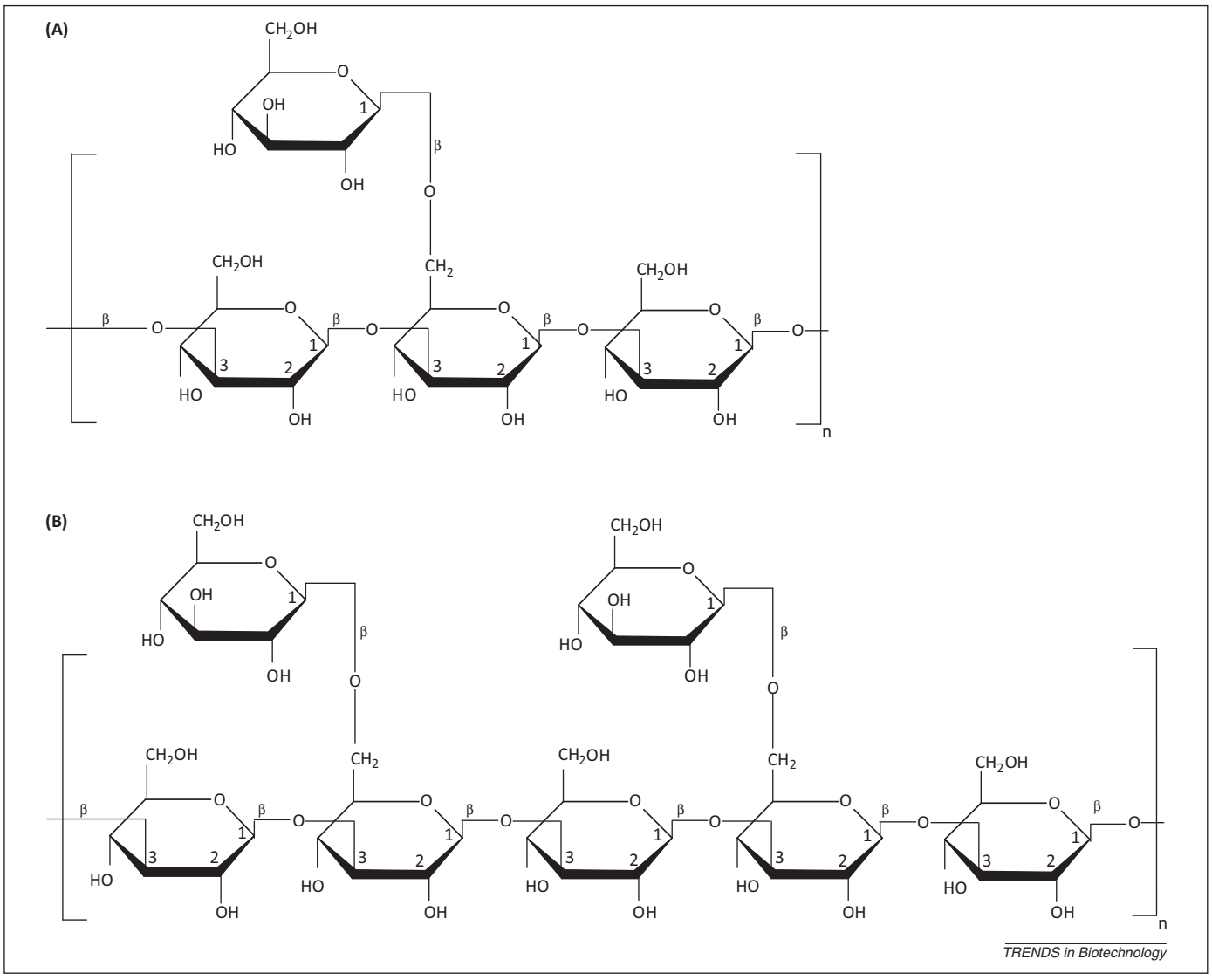

Figure 2. Some examples of polysaccharides produced by mushroom of immunomodulating activities. **(A)** Pleuran (CAS No. 159940-37-1) is an insoluble polysaccharide [β-(1,3/1,6)-D-glucan], isolated from *Pleurotus ostreatus*. **(B)** Lentinan (CAS No. 37339-90-5) is a polysaccharide isolated from the fruiting body of shiitake (*Lentinula edodes*). Lentinan is a β-1,3 glucan with β-1,6 branching.

polysaccharides with random coil conformation can also exhibit potent immunomodulating and anticancer activity [46].

Chemical modifications of polysaccharides

With an aim to increase the biological activity, chemical modification of the polysaccharides to produce carboxymethylated, hydroxylated, formylmethylated, aminoethylated, or sulfated derivatives has been attempted. The hydroxylated form of schizophyllan, for example, was reported to stimulate the production of NO and TNF-α in macrophages better than the native polysaccharides [47]. Also the sulfated (1-3)-α-D-glucan, a native cell wall glucan from *L. edodes*, exhibited higher immunomodulatory and anticancer activity compared to the native polysaccharides [48]. Another example is the improved immunostimulating activity after sulfonation of (TAPA1), a polysaccharide extracted from *Tremella aurantialba* fruiting bodies [49]. A carboxymethylated-sulfated β-(1-3)-D-glucan from *Po. cocos* showed five times greater anticancer activity compared to the native polysaccharides. The induction of carboxymethyl and sulfate groups increased the possible contact between the modified polysaccharides with the immune cell receptor through hydrogen binding and electrostatic attraction and thus increased the immunological response. This was also proven by the increase in phagocyte and thymus indexes, spleen index, and antibody production. [50]. However, other researchers reported that the high stimulatory effect of the hyper branched β-glucan TM3b after sulfation was mainly attributed to the increase in solubility of the molecule [51]. Thus molecular weight, branching, chemical configuration, and chemical modification have strong influence on the solubility of the polysaccharide, which also reflect directly on molecule bioactivity. Thus, all these polysaccharide features should be studied in an inter-related context for better understanding of the immunomodulatory potency of the compounds.

Medical applications

In spite of many *in vitro* and *in vivo* studies performed during the past 50 years revealing the potential of

Table 2. Mushroom polysaccharides and their immunomodulator activities

Mushroom Latin name	Common names	Biomaterial	Active compound	Immunomodulatory activity	Refs
Agaricus blazei (Agaricus subrufescens)	Royal sun agaricus, Almond portobello	FB, CB	Glycoprotein (ATOM), β-1, 3-D-glucan, with β-1,6-D-glucan branch	Induction of TNF, IFN-γ, and IL-8 production	[68]
Antrodia camphorata	Poroid brown-rot fungus	M	β-1,3-D-Gluco-pyranans with β-1,6-D-glucosyl branches, proteoglycan	Induction of INF-γ, TNF-α	[69]
Cordyceps sinensis	Caterpillar fungus	FB, SM, CB	β-D-glucan, heteroglycan, cordyglucan	Increase in IL-5 induction with decrease in IL-4 and IL-17	[70]
Cryptoporus volvatus	Grey-Brown Sap Rot	FB	β-1,3-D-Glucan	Decrease in TLR2 and activate NF-κB	[71]
Flammulina velutipes	Golden needle mushroom	FB, SM, CB	Glycoprotein, peptidoglycan, (FVP)	Increase NO, IL-1 production, and TNF-α secretion	[72]
Ganoderma lucidum	Reishi, Ling-zhi, Spirit plant	FB, CB	Ganoderan, Heteroglycan, mannoglucan, glycopeptide	Stimulate TNF-α, IL-1, IFN-γ production, activate NF-κB.	[73]
Grifola frondosa	Hen of the woods, Maitake	FB, CB, SM	Grifolan (1-6-monoglucosyl-branched β-1,3-D-glucan), proteoglycan, heteroglycan, galactomannan	Macrophage activation, induction of IL-1, IL-6, and TNF-α secretion	[74]
Hericium erinaceus	Lion's Mane Mushroom, Bearded Tooth Mushroom, Monkey head Mushroom	FB, CB	Heteroglycan, heteroglycan-peptide, β-1,3 branched-β-1,2-mannan	Induce NO production, increase expression of TNF-α, IL-1β, IL-12	[45]
Inonotus obliquus	Clinker polypore, birch conk, Chaga	FB, SM	β-D-glucan	Enhance expression of IL-1β, IL-6, TNF-α, and iNOS in macrophages	[75]
Lentinus edodes	Shiitake, black forest mushroom, golden oak mushroom	FB, CB	Lentinan, glucan, mannoglucan, proteoglycan	Induces non-specific cytotoxicity in macrophage and enhance cytokine production	[76]
Morchella esculenta	Morel mushroom, sponge morel	FB	Galactomannan, β-1,3-D-glucan	Macrophage activation, activate NF-κB	[77]
Morchella conica	Sponge mushroom	FB, CB	Galactomannan	Induces NO, IL-1β, IL-6 production	[78]
Phellinus linteus	Mesima, Black Hoof Fungus	FB	Acidic polysaccharides	Activation of murine B cells, Induce IL-12 and IFN-γ production Block NF-κB, TNF-α, IL-1α, IL-1β, and IL-4 production	[79]
Pleurotus ostreatus	Oyster mushroom	FB, SM, CB	Pleuran, heterogalactan, proteoglycan	Induce IL-4 and IFN-γ production	[63]
Polyporus umbellatus (Grifola umbellata)	Umbrella polypore	SM, FB	Polysaccharides	Enhances TNF-α, IL-1β, and NO production	[80]
Polystrictus versicolor	Turkey tail	FB, SM, CB	Krestin, heteroglycan, glycopeptide, polysaccharide K (PSK), polysaccharide peptide (PSP)	Stimulate T cell activation, induce IFN-γ and IL-2 production, induce gene expression of cytokines (TNF-α, IL-1, IL-6, IL-8)	[81]
Sarcodon aspratus	Black tiger paw	FB	Fucogalactan, 1,6-α-D-glucopyranosyl residue	Increase the release of TNF-α and NO in macrophage	[82]
Schizophyllum commune	Split Gill	FB, WM	Schizophyllan, 1,6-monoglucosyl branched β-1, 3-D-glucan	Activation of T cell, increase interleukin, and TNF-α production	[83]
Sclerotinia sclerotiorum	White mold	FB	Scleroglucan (1,6-monoglucosyl-branched β-1, 3-D-glucan)	Stimulates lymphocyte proliferation, increase release of TNF-α	[40]
Sparassis crispa	Rooting cauliflower mushroom	FB	β-Glucan	Enhances IL-6 and INF-γ production	[84]
Tremella aurantialba	The golden Tremella	FB	Heteroglycan	Enhances mouse spleen lymphocyte proliferation	[49]
Xylaria nigripes	Dead moll's fingers	WM	β-Glucan	Inhibits NO, IL-1β, IL-6, TNF-α, and IFN-γ production	[85]

Abbreviation: M, mycelium; WM, whole mushroom; SM, submerged mycelium; CB, culture broth; FB, fruiting bodies.

mushroom extracts as immunomodulators, little information is available on their efficacy in clinical trials. The mushroom polysaccharides of defined chemical structure such as lentinan, schizophyllan, and grifolan have been used in clinical trials. Lentinan was the first approved mushroom polysaccharide for clinical use as an immune adjuvant to chemotherapy for stomach cancer treatment in Japan since 1985 [52]. Nowadays, mushroom

polysaccharides are available in the market as nutraceuticals under different brand names such as Ganodex, Immuna, Lentinex, Immunoglukan, Pure Red Reishi Capsule, LifeShield, Bene-X, and Zymucan. These products are widely used as adjuvants to cancer radio- and chemotherapy, antibiotics, and vaccines to minimize their side effects. Recent research shows that pleuran-based nutraceutical product (Immunoglukan P4H) reduces morbidity caused by recurrent respiratory tract infections through the modulation of humoral and cellular immunity [53].

The anticancer activity of mushroom polysaccharides is mediated through the activation of the immune system

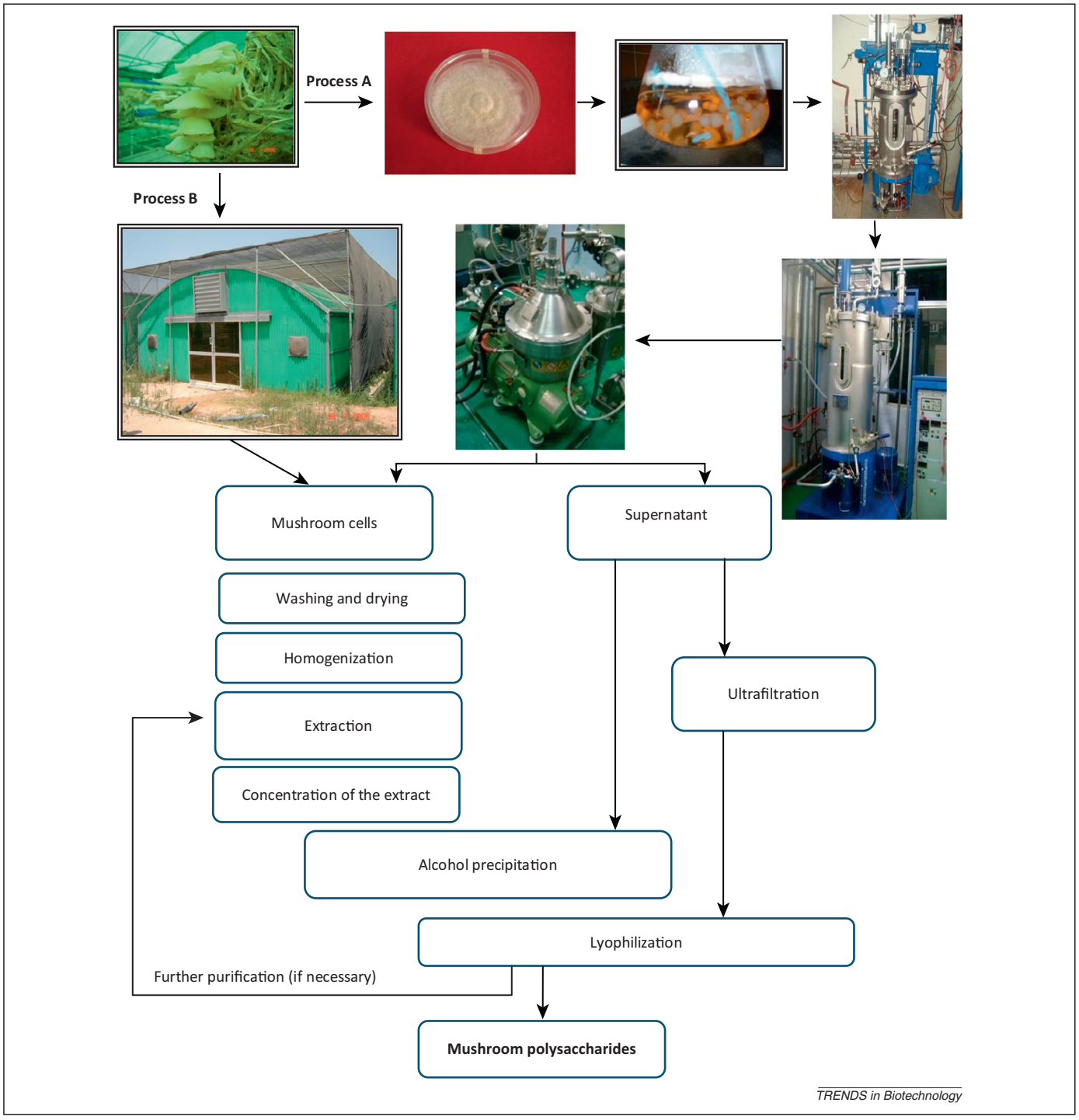

Figure 3. Process for pleuran production by *Pleurotus ostreatus* using submerged mycelium fermentation (Process A) or solid state fermentation (Process B). Process A begins with mushroom cell propagation on agar plates followed by submerged cultivation of cells in shake flasks, as an intermediate step for inoculum preparation for bioreactor culture. Cultivation in the bioreactor is carried out by scaling up the process from a small to a large bioreactor followed by centrifugation to separate cells from the culture broth. If the polysaccharides produced are extracellular, extraction is usually carried out either through direct precipitation using cold ethanol followed by lyophilization to obtain the crude extract. A solvent-free method was also developed to separate high molecular weight compounds directly by ultrafiltration followed by direct lyophilization of the polysaccharides. The endopolysaccharides are extracted using the conventional scheme of polysaccharides extraction from mushroom fruiting bodies (Process B). With fruiting bodies as starting materials (Process B), cells are first washed and dried followed by homogenization prior to several hours of extraction with boiling water. The water extract of crude polysaccharide is concentrated using vacuum extraction followed by alcohol precipitation using cold ethanol. The precipitate is then lyophilized. In this process, further purification could be also done by repeating the steps between water extraction step and lyophilization.

[46,54]; however, other mechanisms such as direct inhibition of cancer cell growth (apoptotic and necrotic effect) can also contribute. In addition to the above applications, mushroom immunomodulators are currently used in cosmeceutical industries and have become basic ingredients in functional creams for wound repair and antiaging [55].

Applications in animal feed and aquaculture

Different types of antibiotics have been used widely as integral parts of animal feed systems to improve growth, control microbial infections, and decrease mortality during the spread of epidemic disease and the early stage of animal growth. However, with the increasing threat of antibiotic-resistant pathogens, their application has been banned in Europe since 2006 [Regulation (EC) No. 1831/2003] [56]. Therefore, replacement of antibiotics with natural immunomodulators to boost natural defense has been of interest [57]. Being a high volume–low value market, most research has focused on using whole mushroom or crude extracts as animal or aquaculture feed additives. Recently, mushrooms were added as feed additives either alone or in combination with probiotics to enhance the immune system in broiler chickens [58].

The immunostimulatory effect of mushroom was mainly attributed to the enhancement of probiotic flora growth, limiting the growth of microbial pathogens. Shiitake mushroom extract has been used to boost the immune system of chickens through direct activation of lymphocytes and macrophages; the level of mRNAs encoding IL-1β, IL-6, IL-12, and IL-18 in macrophages was enhanced [59]. In aquaculture application, diet enrichment with the black hoof fungus (*Phellinus linteus*) extract enhanced the disease resistance of kelp grouper fish against pathogens such as *Vibrio harveyi, Vibrio alginolyticus*, and *Vibrio carchariae*; the resistance was mediated through enhancement of cellular and humoral immune response components such as serum lysozyme activity, and phagocytic activity [60]. The immunostimulatory potential of lentinan extract for healthy growth of sea cucumber (*Apostichopus japonicus*) was reported recently, which was mediated through the increase of phagocytic activity and viability of coelomocytes [61]. A significant increase in immune response and *Vibrio* disease resistance of white shrimp (*Litopenaeus vannamei*) was obtained using feed supplemented with small particle of monkey head mushroom *Hericium erinaceum*. This was due to the increase in phenoloxidase, superoxide dismutase and glutathione peroxidase activities. Recently, it was shown that administration of β-glucan mixture enhances the innate immune response in the high value orange spotted grouper fish (*Epinephelus coidoides*) and protects it against *V. alginolyticus* [62].

Industrial production of mushroom immunomodulators

Mushrooms were first cultivated using hardwood tree logs as a substrate; producing high levels of lignocellulosic hydrolytic enzymes, mushrooms are able to use wood components as nutrition source. This process occurs over several years and yields only two crops per year. Subsequently, solid state fermentation (SSF), in which mushrooms grow on a combination of compost, wood residue, and minerals in polypropylene bags, became more common in mushroom farms. After autoclaving, the bags are inoculated by actively growing mycelia and incubated in greenhouses. After a long cultivation time, sometimes exceeding 2 months, the fruiting bodies are harvested and used for extraction and purification of the bioactive compound. In SSF, the substrates can vary based on mushroom type. In this process, temperature and humidity should be carefully controlled, and the greenhouse is kept clean to reduce the risk of contamination with pathogens. However, for production of mushroom immunomodulators for medicinal applications, the process must be in compliance with current good manufacturing practice (cGMP) for active pharmaceutical ingredients (APIs), which is difficult to implement in SSF given that it is an open cultivation system; there are many challenges including the quality of substrate materials, cultivation parameters and controlled yield of the desired compound. Thus, in recent years research has focused on mushroom cultivation in a closed system using submerged fermentation (SMF). Its advantages include a higher yield of active metabolite in a shorter time, total control of all cultivation parameters, cultivation under fully sterile conditions, and easier downstream processes. Figure 3 shows a simple production platform for mushroom polysaccharides using SSF and SMF systems. Like other fungal fermentations, this process is governed by several factors including medium composition, cultivation conditions such as pH, temperature, aeration rate, and agitation, and fungal morphology in the cultivation vessel [63–66]. The type and concentration of carbon and nitrogen sources applied, C/N ratio, and addition of trace elements/supplements strongly affect polysaccharide biosynthesis. Moreover, oxygen supply acts as one of the bottleneck parameters affecting the yield of polysaccharides production. Fed-batch cultivation seems to be the most appropriate method for high polysaccharides yield as in case of *Agaricus brasiliensis* and *Pleurotus ostreatus* [67].

Concluding remarks

This review has highlighted the importance of mushrooms as future biofactories for the production of immunomodulator compounds of highly diverse chemical structures with potential applications in human and animal health. However, the application of mushroom immunomodulators in modern medicine faces six main challenges that need to be addressed. First, most mushrooms are difficult to cultivate in greenhouses; their availability is seasonal and weather dependent. Second, the content of bioactive ingredients depends on the collection time, procedure, season, and environment. Third, the mushroom greenhouse cultivation method is not validated and not run according to the cGMP requirements. Therefore, more research is needed for the development of cultivation processes in submerged culture to produce bioactive metabolites for pharmaceutical applications. Fourth, mushrooms are slow growing and produce bioactive compounds in low concentrations. This could be overcome by expressing the relevant genes in other hosts with higher growth rate and productivity such as *E. coli*, *P. pastoris*, and the fungus *Aspergillus niger*. Fifth, in most of the research conducted thus far, the immunomodulatory activities were assayed using crude mushroom extracts or

mixtures of different metabolites. It will be necessary to isolate and identify the active metabolites for better understanding of the immunomodulatory mechanism of each particular compound. Upgrading the crude extract from the nutraceutical to the pharmaceutical market will require careful product formulation and clinical trials to determine the proper dose and prove efficacy. Finally, there is a lack of standard testing protocols to guarantee the quality and the efficacy of the mushroom products for pharmaceutical applications. Some mushroom compounds, such as polysaccharides, are highly diversified in terms of structure and molecular weight; thus, it is difficult to produce the same quality from batch to batch. Increasing demand for these natural products will drive development towards overcoming these barriers for entering the market.

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